AN INVESTIGATION INTO THE NEUROPROTECTIVE AND NEUROTOXIC PROPERTIES OF LEVODOPA, DOPAMINE AND SELEGILINE

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ABSTRACT

Parkinson's disease (PD) is a neurodegenerative disorder characterized by a profound loss of dopaminergic neurons from the *substantia nigra* (SN). Among the many pathogenic mechanisms thought to be responsible for the demise of these cells, dopamine (DA)-dependent oxidative stress and oxidative damage has taken center stage due to extensive experimental evidence showing that DA-derived reactive oxygen species (ROS) and oxidized DA metabolites are toxic to SN neurons. Despite its being the most efficacious drug for symptom reversal in PD, there is concern that levodopa (LD) may contribute to the neuronal degeneration and progression of PD by enhancing DA concentrations and turnover in surviving dopaminergic neurons. The present study investigates the potential neurotoxic and neuroprotective effects of DA *in vitro*. These effects are compared to the toxicity and neuroprotective effects observed in the rat striatum after the administration of LD and selegiline (SEL), both of which increase striatal DA levels. The effects of exogenous LD and/or SEL administration on both the oxidative stress caused by increased striatal iron (II) levels and its consequences have also been investigated.

6-Hydroxydopamine (6-OHDA) is a potent neurotoxin used to mimic dopaminergic degeneration in animal models of PD. The formation of 6-OHDA *in vivo* could destroy central dopaminergic nerve terminals and enhance the progression of PD. Inorganic studies using high performance liquid chromatography with electrochemical detection (HPLC-ECD) show that hydroxyl radicals can react with DA to form 6-OHDA *in vitro*. SEL results in a significant decrease in the formation of 6-OHDA *in vitro*, probably as a result of its antioxidant properties. However, the exogenous administration of LD, with or without SEL, either does not lead to the formation of striatal 6-OHDA *in vivo* or produces concentrations below the detection limit of the assay. This is despite the fact that striatal DA levels in these rats are significantly elevated (two-fold) compared to the control group.

The auto-oxidation and monoamine oxidase (MAO)-mediated metabolism of DA causes an increase in the production of superoxide anions in whole rat brain homogenate *in vitro*. In addition to this, DA is able to enhance the production of hydroxyl radicals by Fenton chemistry (Fe(III)-EDTA/H_2O_2) in a cell free environment. Treatment with systemic LD elevates the production of striatal superoxide anions, but does not lead to a detectable increase in striatal hydroxyl radical production *in vivo*. The co-adminstration of SEL with LD is able to prevent the LD induced rise in striatal superoxide levels.

It has been found that the presence of DA or 6-OHDA is able to reduce lipid peroxidation in whole rat brain homogenate induced by Fe(II)-EDTA/H_2O_2 and ascorbate (Fenton system). However, DA and 6-OHDA increase protein oxidation in rat brain homogenate, which is further increased in the presence of the Fenton system. In addition to this, the incubation of rat brain homogenate with DA or 6-OHDA is also accompanied by a significant reduction in the total GSH content of the homogenate. The exogenous administration of LD and/or SEL was found to have no detrimental effects on striatal lipids, proteins or total GSH levels. Systemic LD administration actually had a neuroprotective effect in the striatum by inhibiting iron (II) induced lipid peroxidation.

Inorganic studies, including electrochemistry and the ferrozine assay show that DA and 6-OHDA are able to release iron from ferritin, as iron (II), and that DA can bind iron (III), a fact that may easily impede the availability of this metal ion for participation in the Fenton reaction. The binding of iron (III) by DA appears to discard the involvement of the Fenton reaction in the increased production of hydroxyl radicals induced by the addition of DA to mixtures containing Fe(II)-EDTA and hydrogen peroxide. 6-OHDA did not form a metal-ligand complex with iron (II) or iron (III). In addition to the antioxidant activity and MAO-B inhibitory activity of SEL, the iron binding studies show that SEL has weak iron (II) chelating activity and that it can also form complexes with iron (III). This may therefore be another mechanism involved in the neuroprotective action of SEL.

The results of the pineal indole metabolism study show that the systemic administration of SEL increases the production of N-acetylserotonin (NAS) by the pineal gland. NAS has been demonstrated to be a potent antioxidant in the brain and protects against 6-OHDA induced toxicity.

The results of this study show that DA displays antioxidant properties in relation to lipid peroxidation and exhibits pro-oxidant properties by causing an increase in the production of hydroxyl radicals and superoxide anions, as well as protein oxidation and a loss of total GSH content. Despite the toxic effects of DA *in vitro*, the treatment of rats with exogenous LD does not cause oxidative stress or oxidative damage. The results also show that LD and SEL have some neuroprotective properties which make these agents useful in the treatment of PD.

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%	percentage
<	less than
>	greater than
µCi	microcurie
µg	micrograms
µl	microliters
µm	micrometers
µM	micromolar (micromoles per liter)
•OH	hydroxyl radical
2,3-DHBA	2,3-dihydroxybenzoic acid
2,4-DNPH	2,4-dinitrophenylhydrazine
2,5-DHBA	2,5-dihydroxybenzoic acid
3-MT	3-methoxytyramine
5-HIAA	5-hydroxyindole acetic acid
5-HT	serotonin
5-HTOH	5-hydroxytryptophol
5-MIAA	5-methoxyindole acetic acid
5-MT	5-methoxytryptamine
5-MTOH	5-methoxytryptophol
6-OHDA	6-hydroxydopamine
6-OHDA-Q	6-hydroxydopamine quinone
A	amperes
AADC	aromatic amino acid decarboxylase
AC	anodic current
ACN	acetonitrile
AdSV	adsorptive stripping voltammetry
Ag	silver
AgCl	silver chloride

ANOVA	analysis of variance
ATP	adenosine triphosphate
BBB	blood brain barrier
bd	twice daily
BHT	butylated hydroxytoluene
BSA	bovine serum albumin
Ca^{2+}	calcium ions
cAMP	cyclic adenosine monophosphate
CAT	catechol
CNS	central nervous system
CO	carbon monoxide
COMT	catechol-O-methyltransferase
COX-1	cyclooxygenase-1
COX-2	cyclooxygenase-2
CSF	cerebrospinal fluid
CV	cyclic voltammetry
Cys	cysteine
CYT-C	cytochrome c
CYT-C-OX	cytochrome c (oxidized form)
CYT-C-RED	cytochrome c (reduced form)
DA	dopamine
DA-Q	dopamine quinone
DAT	dopamine transporter
DBH	dopamine β-hydoxylase
Dcytb	duodenal ferric reductase
DHBT-1	7-(2-aminoethyl)-3,4-dihydro-5-hydroxy-2H-1,4-benzothiazine-3-carboxylic acid
DMT-1	divalent metal transporter 1
DNA	deoxyribonucleic acid
DOPAC	3,4-dihydroxyphenylacetic acid
DOPAC-Q	3,4-dihydroxyphenylacetic acid quinone

DOPALD	3,4-dihydroxyphenylacetaldehyde
DPM	disintegrations per minute
DTNB	5,5-dithiobis-(2-nitrobenzoic acid)
$E_{1/2}$	half-wave potential
EDTA	ethylenediaminetetraacetic acid
Ep(a)	peak electrical potential
ESR	electron spin resonance
Fe^{2+}	ferrous iron
Fe^{3+}	ferric iron
$FeCl_3$	anhydrous ferric chloride
GABA	γ-aminobutyric acid
GCE	glassy carbon electrode
GCS	γ-glutamyl cysteine synthase
GIT	gastrointestinal tract
GPe	external segment of the *globus pallidus*
GPi	internal segment of the *globus pallidus*
GP_x	glutathione peroxidase
GR	glutathione reductase
GSH	glutathione (reduced form)
GSSG	glutathione (oxidized form)
H^+	hydrogen ions
H_2O_2	hydrogen peroxide
HSA	heptane sulphonic acid
$HClO_4$	perchloric acid
HIOMT	hydroxyindole-O-methyl transferase
HNE	4-hydroxy-2-nonenal
HOO•	hydroperoxyl radical
HPLC	high performance liquid chromatography
HVA	homovanillic acid
i.p.	intraperitoneal

Ia	current
KCl	potassium chloride
kg	kilogram
L	liters
L•	lipid radical
LD	levodopa
Leukoaminochrome-o-SQ•	leukoaminochrome ortho-semiquinone radical
LH	unsaturated lipid
LIP	labile iron pool
LOD	limit of detection
LOO•	peroxyl radical
LOOH	lipid peroxide
LOQ	limit of quantification
Lys	lysine
M	molar (moles per liter)
M(Ligand)	metal-ligand complex
m/v	mass by volume
MAO	monoamine oxidase
MAO-A	monoamine oxidase-A
MAO-B	monosmine oxidase-B
MDA	malondialdehyde
MEL	melatonin
mg	milligrams
min	minutes
ml	milliliter
mm	millimeters
mM	millimolar (millimoles per liter)
mmol	millimoles
M^{n+}	metal ion
MPP^{+}	1-methyl-4-phenylpuridinium ion

MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
N	nitrogen
nA	nanoamperes
NADH	nicotiamide adenine dinucleotide (reduced form)
NADPH	nicotiamide adenine dinucleotide phosphate (reduced form)
NAS	N-acetylserotonin
NAT	N-acetyltransferase
NBD	nitroblue diformazan
NBT	nitroblue tetrazolium
NE	norepinepherine
NH	amine functional group
nm	nanometers
nmol	nanomoles
NO•	nitric oxide
NOS	nitric oxide synthase
O_2	oxygen
$O_2^{\bullet-}$	superoxide anion radical
^oC	degrees Celsius
OH	hydroxyl functional group
$ONOO^-$	peroxynitrite
PA	phosphoric acid
PBS	phosphate buffered saline
PD	Parkinson’s disease
pmol	picomoles
PNMT	phenylethanolamine N-methyltrasferase
PST	phenolsulfotransferase
PUFA	polyunsaturated fatty acid
redox	reduction-oxidation
ROS	reactive oxygen species

RSD	relative standard deviation
s	seconds
SD	standard deviation
SEL	selegiline
SEL•	selegiline radical
SH	thiol or sulfhydryl group
SN	*substantia nigra*
SNpc	*substantia nigra pars compacta*
SNpr	*substantia nigra pars reticulate*
SOD	superoxide dismutase
SQ•	semiquinone radical
SSRI	selective serotonin reuptake inhibitors
TBA	thiobarbituric acid
TCA	trichloracetic acid
TEA	triethylamine
TH	tyrosine hydroxylase
TLC	thin layer chromatography
Tris-HCl	Tris (hydroxymethyl)-aminomethane hydrochloride
UCH-L1	ubiquitin C-terminal hydrolase L1
UPS	ubiquitin-proteasome system
UQ	coenzyme Q (oxidized form)
$UQ^{\bullet -}$	coenzyme Q (semiquinone anion species)
UQH_2	coenzyme Q (reduced form)
UV	ultraviolet
UV/VIS	ultraviolet and visible
Vit E	vitamin E
Vit E•	vitamin E radical
V	volts
x g	relative centrifugal field

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PUBLICATIONS

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CHAPTER ONE

LITERATURE REVIEW

1.1 NEUROANATOMY

1.1.1 The Basal Ganglia

The basal ganglia represent one of the most important subcortical structures in the motor circuit. It comprises the *caudate nucleus* and *putamen*, which are together known as the corpus striatum, together with the external and internal segments of the *globus pallidus* (GPe and GPi, respectively), the *subthalamic nucleus* and the two divisions of the *substantia nigra*, the *pars reticulate* (SNpr) and the *pars compacta* (SNpc) (figure 1.1) (Connor, 1998; Alexander and Crutcher, 1990; Stein and Stoodley, 2006).

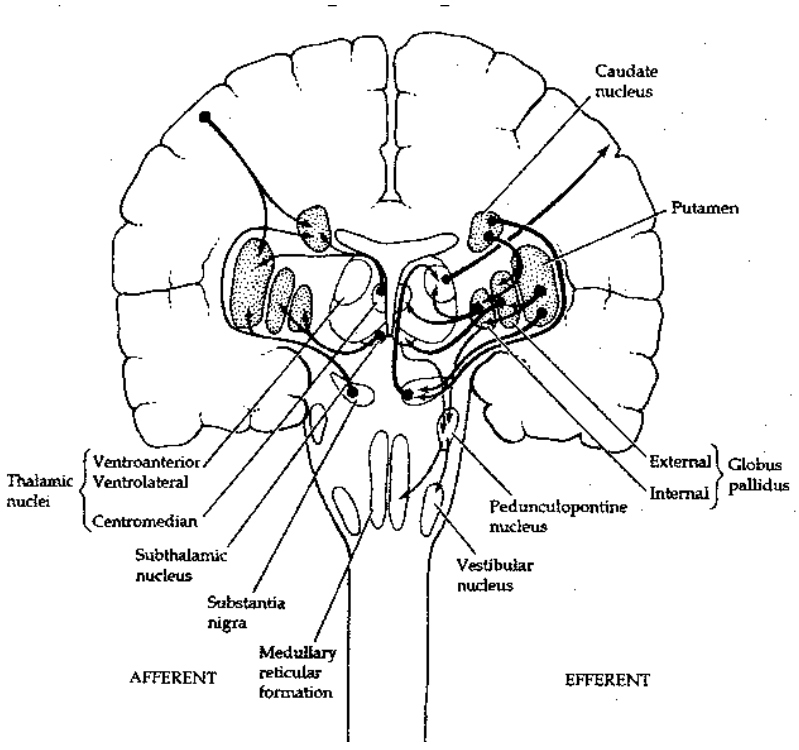


Figure 1.1: The structural components of the basal ganglia.
(http://www.benbest.com/science/anatmind/anatmd2.html)

These brain regions represent processing centers intimately involved in the regulation of movement. Striatal γ-aminobutyric acid (GABA) neurons form a majority (90 %) of the final output of the striatum and comprise two opposite and opposing GABA efferent pathways, namely the "direct pathway" which promotes movement by disinhibiting the motor thalamus and the "indirect pathway" which inhibits movement (figure 1.2).

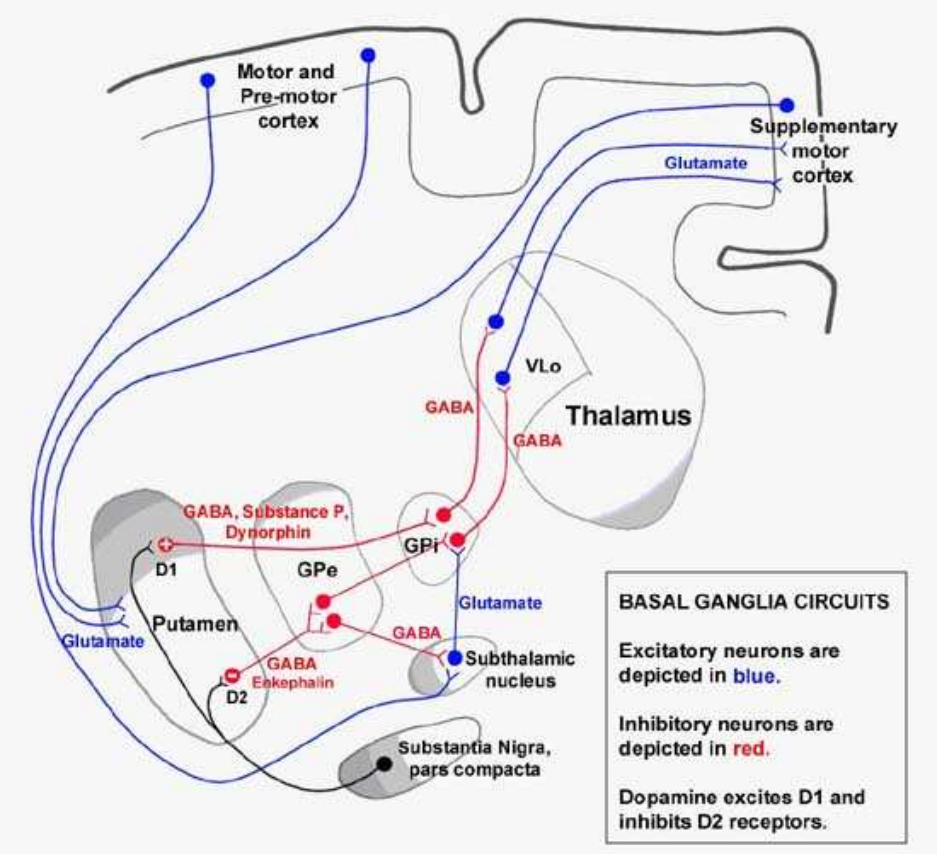


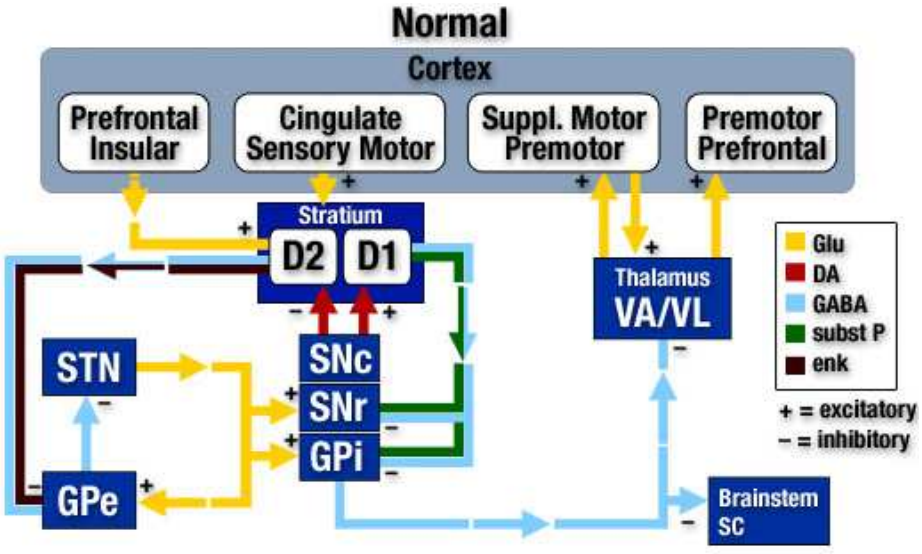
Figure 1.2: Schematic representation of the pathways through the basal ganglia. (http://medinfo.ufl.edu/year2/neuro/review/gang.html)

In the direct pathway, the neocortex glutaminergically excites the *putamen*. This in turn GABA inhibits (with substance P as the co-transmitter) the GPi and the SNpr. As a result of this the normal GPi GABA inhibition of the thalamus and reticular formation (RF) is turned off. Any ongoing activity in the thalamus and RF is therefore facilitated, promoting movement. In the indirect pathway, the neocortex glutaminergically excites the *caudate* and this then GABA inhibits (with enkephalin as co-transmitter) the GPe.

This inhibition prevents the GPe from GABA inhibiting the subthalamic nucleus. This excites (glutaminergically) the GPi and SNpr. Hence, these nuclei increase their inhibition of thalamocortical and brainstem movements (Stein and Stoodley, 2006).

Both the direct and indirect pathways receive dopaminergic afferents from the SNpc (William, 1998). This is the nigrostriatal dopaminergic pathway which degenerates in patients with Parkinson's disease (PD), one of the most common neurodegenerative diseases. Dopamine (DA) acts as a neuromodulator in the striatum, where it is released from axonal terminals and in the SNpr where it is released from pars compacta dendrites. In the putamen it enhances glutamate signals from the cerebral cortex if these reach a certain depolarizing threshold, but suppresses background signals that do not reach this threshold. DA therefore acts as a sort of neural "filter" by enhancing the recruitment of appropriate movement programmes, while inhibiting irrelevant background noise. The mechanism by which DA acts as a neural "filter" is, at least in part, attributed to excitatory D_1 receptors that increase cAMP on the direct, facilitatory pathway through the striatum, but inhibitory D_2 receptors that decrease cAMP on the inhibitory pathway via the GPe. In PD, therefore, the lack of DA in the striatum means that the direct route is depressed and the in-direct route is facilitated. The main effect is therefore over-inhibition of thalamic and reticular motor programmes by the GPi, resulting in akinesia (figure 1.3)(Stein and Stoodley, 2006).

A



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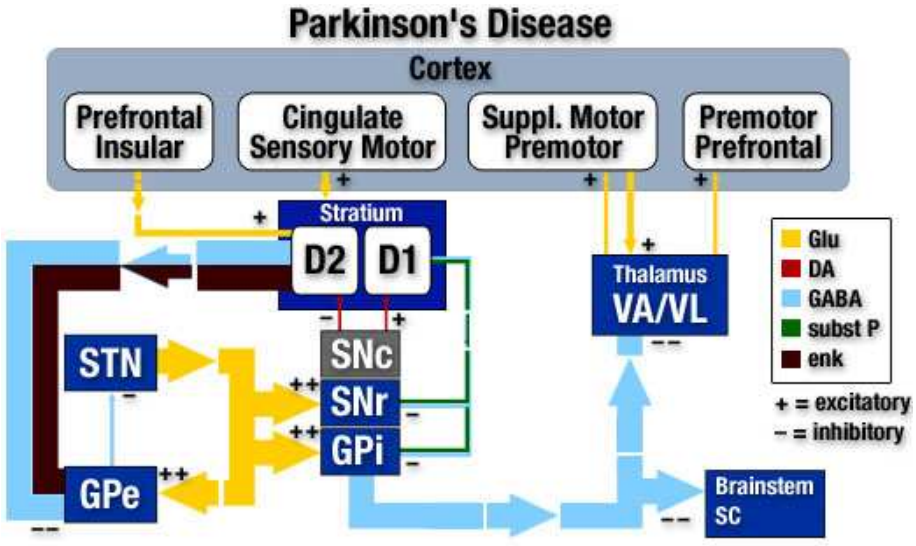

Figure 1.3: (A) Normal functional anatomy of the basal ganglia and (B) Pathological functional anatomy of the basal ganglia. The arrows point to the direction of the different nerve tracts and the colors indicate the neurotransmitters involved at each level. The widths of the tracts are proportional to the strength of the signal. Positive signs at the end of a nerve tract indicate excitatory impulses and negative signs indicate inhibitory impulses. (http://www.mdvu.org/library/disease/pd/par_path.html)

1.2. OXIDATIVE STRESS

1.2.1 Introduction

Although aerobic organisms need oxygen for survival, in many cases the use of oxygen by these organisms leads to their slow demise. This is known as the oxygen paradox (Halliwell and Gutteridge, 1984). Oxidative stress is the cytotoxic consequence of oxyradical and oxidant formation and reaction with cellular components. The oxygen species that are typically linked to oxidative stress are the superoxide anion radical ($O_2^{\bullet -}$), hydroxyl radical (•OH), hydrogen peroxide (H_2O_2), nitric oxide (NO•) and peroxynitrite ($ONOO^-$). The collective term used for these chemicals is "reactive oxygen species" (ROS), however, some of these species are not very reactive in biological solutions (Dawson and Dawson, 1996).

Levels of ROS in excess of the normal needs of the cell may indiscriminately damage the structural and functional integrity of the cell. This they do by directly modifying cellular DNA, proteins and lipids, or by initiating radical chain reactions that can cause extensive oxidative damage to these critical biomolecules. Although cells possess a variety of antioxidant defense mechanisms against ROS, this can sometimes be inadequate leading to oxidative stress in which the production of ROS overwhelms the antioxidant defenses of the organism (Cui *et al*., 2004). Oxidative stress can therefore be regarded as an imbalance between oxidative events and opposing antioxidant defenses (Fahn and Cohen, 1992).

1.2.2 Reactive oxygen species

The toxicity of oxygen has been attributed to the formation of toxic species generated by the partial reduction of oxygen. These toxic species are often referred to as reactive oxygen species (ROS). The term ROS encompasses all reactive oxygen species, both the radical and nonradical species involved in the initiation and/or propagation of free radical chain reactions (Dawson and Dawson, 1996). ROS include the superoxide radical ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2) and hydroxyl radicals (•OH). Some of these species contain unpaired electrons and are therefore referred to as free radicals (Halliwell, 1992).

1.2.2.1 Superoxide radical

The acceptance of a single electron by O_2 results in the formation of the superoxide radical ($O_2^{\bullet-}$). A major source of $O_2^{\bullet-}$ is the mitochondrial electron transport chain (Reiter, 1998). The generation of superoxide radicals at the level of cytochrome c oxidase is virtually nonexistent (Kowaltowski and Vercesi, 1998). However, electron leakage to molecular oxygen by intermediate electron carriers in the electron transport chain can result in the monoelectronic reduction of O_2 to $O_2^{\bullet-}$ (Halliwell, 1992). This may occur at the level of NADH dehydrogenase or at the level of coenzyme Q (Kowaltowski and Vercesi, 1998). Once $O_2^{\bullet-}$ has been produced it is readily dismutated to hydrogen peroxide by a group of enzymes that scavenge $O_2^{\bullet-}$ called the superoxide dismutates (SOD) (Fridovich, 1989). SOD is considered an important antioxidative enzyme, since it removes $O_2^{\bullet-}$ from cells (Reiter, 1998).

Superoxide radicals are also formed by the auto-oxidation of DA, epinephrine and norepinephrine catalysed by transition metal ions, and by the action of enzymes such as indoleamine dioxygenase, tryptophan hydroxylase and xanthine oxidase (Cui *et al*., 2004).

Superoxide itself has limited reactivity towards biological substrates and the number of molecular targets that are known to be sensitive to $O_2^{\bullet-}$ are small. In some circumstances

the controlled generation of $O_2^{\bullet-}$ can be beneficial. Activated phagocytes produce $O_2^{\bullet-}$ which plays an important role in the way bacteria are engulfed and destroyed by these cells (Colton and Gilbert, 1987). Monocytes, neutrophils, eosinophils and various macrophages including microglial cells in the brain are all capable of generating $O_2^{\bullet-}$ (Colton *et al*., 1994). Small amounts of $O_2^{\bullet-}$ are also produced and released by other cell types such as fibroblasts, lymphocytes and endothelial cells during normal physiological reactions. The $O_2^{\bullet-}$ generated by these cells may play a role in the regulation of growth as well as intercellular signaling (Halliwell, 1992).

Superoxide is capable of inactivating certain enzymes such as creatine kinase in mammals and some iron-sulphur proteins in bacteria. Superoxide can also inactivate the NADH dehydrogenase complex of the mitochondrial electron transport chain *in vitro* (Zhang *et al*., 1990). However, this has not yet been demonstrated *in vivo* (Halliwell, 1992). The inappropriate mobilization of iron from ferritin is another mechanism by which $O_2^{\bullet-}$ can cause damage to living systems (Williams *et al*., 1974; Biemond *et al*., 1984).

In solution, $O_2^{\bullet-}$ exists in equilibrium with the hydroperoxyl radical (HOO•). HOO• is a much stronger oxidant than $O_2^{\bullet-}$ and will directly attack polyunsaturated fatty acids (Bielski *et al*., 1983). HOO• is also more readily dismutated to H_2O_2 than is $O_2^{\bullet-}$ (Reiter, 1998).

1.2.2.2 Hydrogen peroxide

As already mentioned $O_2^{\bullet-}$ is readily removed from cells by its conversion into H_2O_2 by SOD. Other enzymes capable of generating H_2O_2 include L-amino acid oxidase, glycolate oxidase and monoamine oxidase (Sies, 1991). All these enzymes like the SODs are also found in human tissues.

The main catabolic pathway for DA in dopaminergic nerve terminals is its deamination by MAO, leading to H_2O_2 generation (Halliwell, 1992; Ogawa and Mori, 1995). It has

been proposed that an accelerated turnover of DA within dopaminergic nerve terminals of Parkinson's patients may lead to an increase in H_2O_2 production. This in turn could accelerate the destruction of these dopaminergic neurons by giving rise to an increase in reactive oxygen species beyond the ability of the brains antioxidant defenses to cope with them. Similarly, the side effects of long term L-Dopa (LD) treatment could be caused by excessive formation of H_2O_2 and its conversion into more toxic species (Olanow, 1990).

H_2O_2 is known to be toxic to many systems, including the nervous system. However, its toxicity is usually not attributed to H_2O_2 itself (except in high concentrations), but rather to the conversion of H_2O_2 into highly oxidizing cell damaging radicals. H_2O_2 does not qualify as a radical because it does not have any unpaired electrons (Halliwell, 1992).

Unlike $O_2^{\bullet-}$ which is a charged molecule, H_2O_2 is electrically neutral. As a result of this H_2O_2 can diffuse through cell membranes more easily than $O_2^{\bullet-}$. Thus H_2O_2 can distribute to sites that are distant from the site of its formation. Another hazardous aspect of the H_2O_2 molecule is that it is able to generate highly reactive hydroxyl radicals (•OH) in the presence of transition metal ions, most often Fe^{2+}. The univalent reduction of H_2O_2 to •OH by metal ions occurs via either the Fenton reaction (equation 1.1) or the Haber-Weiss reaction (equation 1.2) (Imlay *et al*., 1988; Halliwell and Gutteridge, 1990; Yamasaki and Piette, 1991).

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + \bullet OH + OH^- \text{ (Fenton reaction)} \qquad \text{(Equation 1.1)}$$

$$O_2^{\bullet-} + H_2O_2 \xrightarrow{\text{Fe catalyst}} \bullet OH + OH^- + O_2 \quad \text{(Haber-Weiss reaction)} \qquad \text{(Equation 1.2)}$$

H_2O_2 can be removed from cells by the action of two types of enzyme, namely catalases and selenium-dependent glutathione peroxidase (GP_x). The latter enzyme type is probably more important in the brain due to the low activity of catalase in most parts of the central nervous system (CNS) (Jain *et al*., 1991).

1.2.2.3 Hydroxyl radical

The •OH is probably not the only highly oxidizing tissue-damaging species formed during the Fenton reaction (Bielski, 1991). However, its formation is well established as is its ability to react at great speed with almost every molecule found in living cells (Halliwell and Gutteridge, 1989). The initial product of the reaction of H_2O_2 with Fe^{2+} may be an iron-oxygen complex referred to as *ferryl* which itself is highly oxidizing and decomposes to yield •OH (Halliwell, 1992).

It is well known that as a result of its high reactivity the •OH reacts with any molecule within a few Angstroms of where it is produced, including DNA, membrane lipids and carbohydrates. Its estimated half life at 37 ºC is in the order of $1x10^{-9}$ seconds (Reiter, 1998). As a result of this very short half-life, the direct action of the •OH is restricted to molecules in the immediate vicinity of where it is generated. The cytotoxic action of •OH generated in mammalian cells almost always involves DNA damage (Cochrane, 1991). This DNA damage can occur in at least two ways. Firstly, the DNA damage may be mediated by the reaction of H_2O_2 with Fe^{2+} and/or Cu^{+} bound to molecules at or close to the DNA, so that when •OH is formed it reacts with the neighboring nucleic acids (Halliwell and Aruoma, 1989). Alternatively, DNA damage can be caused by an increase in intracellular free calcium concentration brought about by oxidative stress. This in turn leads to the activation of nuclease enzymes in the nucleus which results in the generation of •OH and subsequent DNA damage (Orrenius *et al*., 1989).

Another destructive effect of •OH is its effects on membrane lipids. The •OH radical is capable of initiating the process of lipid peroxidation by abstracting an allelic hydrogen atom from a polyunsaturated fatty acid (PUFA). This in turn can set off a free radical chain reaction wherein lipid peroxidation is self-propagating.

1.2.2.4 Peroxyl radical

Peroxyl radicals (LOO•) are formed during the process of lipid peroxidation. (Asano *et al*.,1991). Peroxyl radicals can attack membrane proteins and in so doing can damage receptors and enzymes. In addition to this they can also abstract hydrogen atoms from PUFA and re-initiate the process of lipid peroxidation (Halliwell, 1992). Vitamin E is the premier scavenger of LOO• and is therefore referred to as a chain breaking antioxidant (Packer, 1994).

1.2.3 Molecular targets of oxidative stress

Oxidative stress can result in deleterious oxidation of numerous biomolecules e.g. lipids, proteins and DNA. Hence, failure of some researchers to find lipid peroxides in injured nervous tissue does not exclude the possibility of oxidative damage to other biomolecules such as proteins and DNA (Orrenius *et al*., 1989; Cochrane, 1991).

1.2.3.1 Lipid peroxidation

The peroxidation of PUFAs is one of the major outcomes of free radical-mediated injury to tissue (Montine *et al*., 2002). PUFAs (those with two or more carbon-carbon double bonds), such as arachidonic acid, are much more sensitive to free radical attack than saturated or monounsaturared fatty acids. Lipid peroxidation of membrane PUFAs may adversely affect membrane fluidity (usually decreasing), peameability (allowing ions such as Ca^{2+} to leak across the membrane), electrical potential, and controlled transport of metabolites across the membrane (Cui *et al*., 2004). Peroxidation of PUFAs, mostly in membrane phospholipids, generally has three well-described phases: initiation, propagation and termination (Montine *et al*., 2002).

Various reactive species, such as •OH are capable of initiating the process of lipid peroxidation by abstracting a hydrogen atom from a methylene group in a PUFA. The abstraction of the hydrogen atom leaves behind a carbon centered radical (L•) in the

membrane which then stabilizes by molecular rearrangement of the double bonds to the conjugated diene form (equation 1.3).

lipid-H + radical → L• + radical-H (initiation) (Equation 1.3)

The most likely fate of the carbon centered radicals is reaction with molecular oxygen to form peroxyl radicals (equation 1.4).

$L\bullet + O_2 \rightarrow LOO\bullet$ (Equation 1.4)

Peroxyl radicals can then attack membrane proteins and in so doing damage receptors and enzymes. In addition to the damage produced by peroxyl radical attack on membrane proteins, the peroxyl radicals can also re-initiate the process of lipid peroxidation by abstracting hydrogen atoms from adjacent PUFAs (equation 1.5).

LOO• + lipid-H → LOOH + L• (propagation) (Equation 1.5)

Lipid peroxidation is therefore extremely damaging because once initiated it is self-propagating since the peroxyl radical can re-initiate the process (Reiter, 1998). This means the abstraction of a single hydrogen atom from a PUFA could set off a free radical chain reaction in which most of the membrane lipids are converted to hydroperoxides (LOOH) and cyclic peroxides.

Transition metals, especially copper and iron ions promote lipid peroxidation in two ways. First, they catalyse the formation of reactive oxygen species capable of initiating lipid peroxidation. Second, they catalyse the decomposition of lipid hydroperoxides to peroxyl radicals and alkoxyl radicals which can propagate lipid peroxidation by abstracting hydrogen atoms (Rikans and Hornbrook, 1997; Halliwell, 1992). Other products of these decomposition reactions include hydrocarbons, alcohols, ethers, epoxides and aldehydes (Cui *et al*., 2004). Of these aldehydes, malondialdehyde and 4-hydroxynonenal have the ability to damage enzymes, proteins, receptors and DNA by resulting in covalent modification (cross-linking) of these molecules (Halliwell, 1992).

Termination of the free radical chain reaction of lipid peroxidation is brought about by reaction between the radicals themselves (equation 1.6), or reaction between the radicals and antioxidants (equation 1.7), giving rise to nonradical products or unreactive radicals.

Lipid• + lipid• → lipid-lipid (termination) (Equation 1.6)

Lipid• + Vit E → lipid + Vit E• (scavenging) (Equation 1.7)

Figure 1.4 depicts the suggested mechanism for the initiation and propagation of lipid peroxidation.

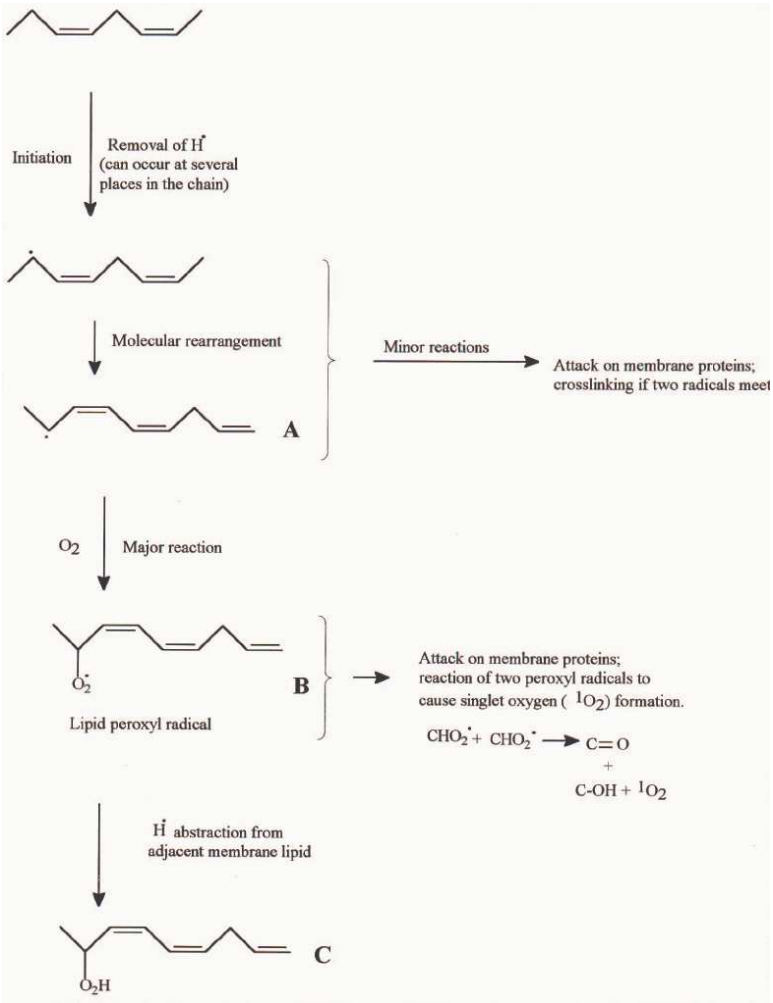

Figure 1.4: An outline mechanism of lipid peroxidation (Gutteridge and Halliwell, 1990).

1.2.3.2 Protein oxidation

The mechanism suggested for free radical induced protein oxidation is shown in figure 1.5. Various reactive species, such as the hydroperoxyl radical (HOO•) or the •OH are capable of intiating the process of protein oxidation by abstracting a hydrogen atom from a β-carbon. Abstraction of a hydrogen atom leaves behind a carbon-centered radical in the protein. The most likely fate of a carbon-centered radical *in vivo* is reaction with oxygen to form a peroxyl radical. This free radical may react with another peroxyl radical to form a tetraoxide (Dean *et al*., 1997) which then breaks down to give an alkoxyl radical and the corresponding side chain radical (Butterfield and Stadtman, 1997). Cleavage of the peptide bond then results in the formation of carbonyl groups that are often used as indicators of protein oxidation (Butterfield and Stadtman, 1997).

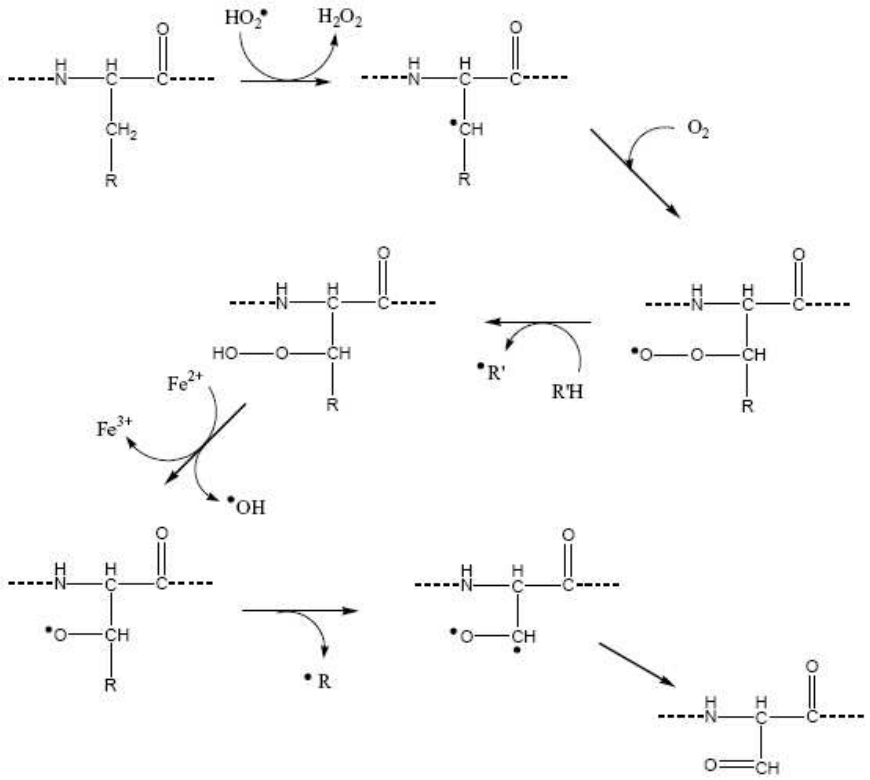

Figure 1.5: Formation of carbonyl groups in proteins following free radical attack (Hermida-Ameijeiras *et al*., 2004).

The iron-mediated oxidation of proteins may be a site-specific process, due to the fact that proline, histidine, arginine, lysine and cysteine residues in proteins are highly sensitive to iron-mediated oxidation (Stadtman, 1990). It is believed that Fe^{2+} binds to metal binding sites on the proteins, and that the Fe(II)-protein complex reacts with H_2O_2 to yield an active oxygen species e.g. •OH, at the site. Protein oxidation can lead to the conversion of some amino acids to carbonyl derivatives, loss of catalytic activity, and increased susceptibility of the protein to proteolytic degradation. It is important to note that protein oxidation can also lead to protein cross-linking in addition to peptide bond cleavage via diamide or α-amidation pathways.

1.2.3.3 DNA oxidation

DNA can be modified by free radicals resulting in single and double-strand breaks, depurination/depyrimidation, or chemical modification of the bases or the sugars (Chevion, 1988). The species responsible for oxidizing DNA is believed to be •OH. Similar to proteins, some researchers postulate that the iron-mediated oxidation of DNA may be a site specific process (Chevion, 1988; Li *et al*., 2001). Iron may bind to the DNA, either to the phosphate backbone or to the purine or pyrimidine bases where the iron can serve as a center for repetitive formation of •OH resulting in modification of the DNA (Giloni *et al*., 1981; Grollman *et al*., 1985).

1.2.4 Antioxidant defense mechanisms

In order to survive in an oxidizing environment, aerobic organisms must be equipped with the necessary molecular tools to combat at least some of the damaging effects caused by excess ROS generation (Reiter, 1998). Excess ROS are usually inactivated by endogenous or exogenous antioxidant molecules (Cui *et al*., 2004). An antioxidant is a substance that even in low concentrations significantly delays or reduces oxidation of a substrate (Gutteridge, 1995). Antioxidants protect other chemicals of the body from oxidation by lowering the local oxygen concentration, removing or reducing the

concentration of species that initiate the oxidative process, such as ROS, chelating transition metals that catalyse the oxidation reactions, interfering with the propagation step of the oxidative process that spreads oxidation to neighboring molecules or by enhancing the endogenous antioxidant defenses of the cell (Cui *et al*., 2004)

Enzyme antioxidants such as superoxide dismutase (SOD), catalase and glutathione peroxidase (GP_x) detoxify specific ROS after they are formed by degrading them to less harmful substances (equation 1.8, 1.9 and 1.10 respectively) (Cui *et al*., 2004).

$$2O_2^{\bullet-} + 2H^+ \xrightarrow{\textbf{SOD}} H_2O_2 + O_2 \qquad \text{(Equation 1.8)}$$

$$2H_2O_2 \xrightarrow{\textbf{Catalase}} O_2 + 2H_2O \qquad \text{(Equation 1.9)}$$

$$H_2O_2 + 2GSH \xrightarrow{\textbf{GP}_\textbf{x}} 2H_2O + GSSG \qquad \text{(Equation 1.10)}$$

SOD provides a first line of defense against oxidative stress by catalyzing the dismutation of superoxide radicals ($O_2^{\bullet-}$) to hydrogen peroxide (H_2O_2) and oxygen (O_2) (equation 1.8) (den Hartog *et al*., 2003). Hydrogen peroxide is not a radical by itself, but is a precursor of the highly reactive hydroxyl radical. Detoxification of H_2O_2 is carried out by both catalase and GP_x. Catalase converts H_2O_2 to oxygen and water (equation 1.9) and GPx reduces H_2O_2 to water in the prescence of glutathione (GSH) (equation 1.10) (Cui *et al*., 2004). GP_x is more important for the detoxification of H_2O_2 in the brain since the brain has reduced catalase activity (Bharath *et al*., 2002).

The preventative antioxidants act by chelating and sequestering transition metal ions, such as iron and copper, which are needed for catalyzing the generation of reactive radical species. Examples of preventative antioxidants are transferrin, lactoferrin and ceruloplasmin (Cui *et al*., 2004). Transferrin and lactoferrin bind iron ions. Iron bound to these proteins is incapable of catalyzing free radical reactions (Halliwell, 1992). Ceruloplasmin has antioxidant properties because most or all of plasma copper is attached to this protein. These copper ions are therefore also unavailable for participating in the generation of reactive radical species (Gutteridge and Stocks, 1981). Isolated

hemoglobin is degraded in the prescence of H_2O_2 resulting in the release of catalytic iron ions from the heme ring. Haptoglobins and hemopexin can therefore also be regarded as preventative antioxidants since they bind hemoglobin and heme respectively (Cui *et al*., 2004).

The chain breaking or scavenging antioxidants interrupt the chain reaction of oxidation induced by reactive radical intermediates by reacting with these intermediates directly and giving rise to products that are unable to propagate the chain further (Cui *et al*., 2004). The main lipid soluble scavengers are vitamin E (α-tocopherol) and β-carotene (Cui *et al*., 2004).Vitamin E (VE) is the principle scavenger of peroxyl radicals ($LOO^{\bullet}$) involved in the propagation of lipid peroxidation (Packer, 1994). Studies have shown that pretreatment of rats with vitamin E attenuates 6-hydroxydopamine toxicity in these animal models of Parkinson's disease (PD) (Gilgun-Sherki *et al*., 2001). The main water soluble scavengers are ascorbic acid, uric acid, various thiols as well as bilirubin (Frei *et al*., 1988). Ascorbic acid has a variety of roles besides the regeneration of vitamin E (Chan, 1993). It also acts as a scavenger of free radicals and inhibits the peroxidation of phospholipids in membranes (Path, 1990).

Enzymatic and nonenzymatic antioxidant defense systems do not function independently of each other but rather function co-operatively in the form of a cascade (figure 1.6).

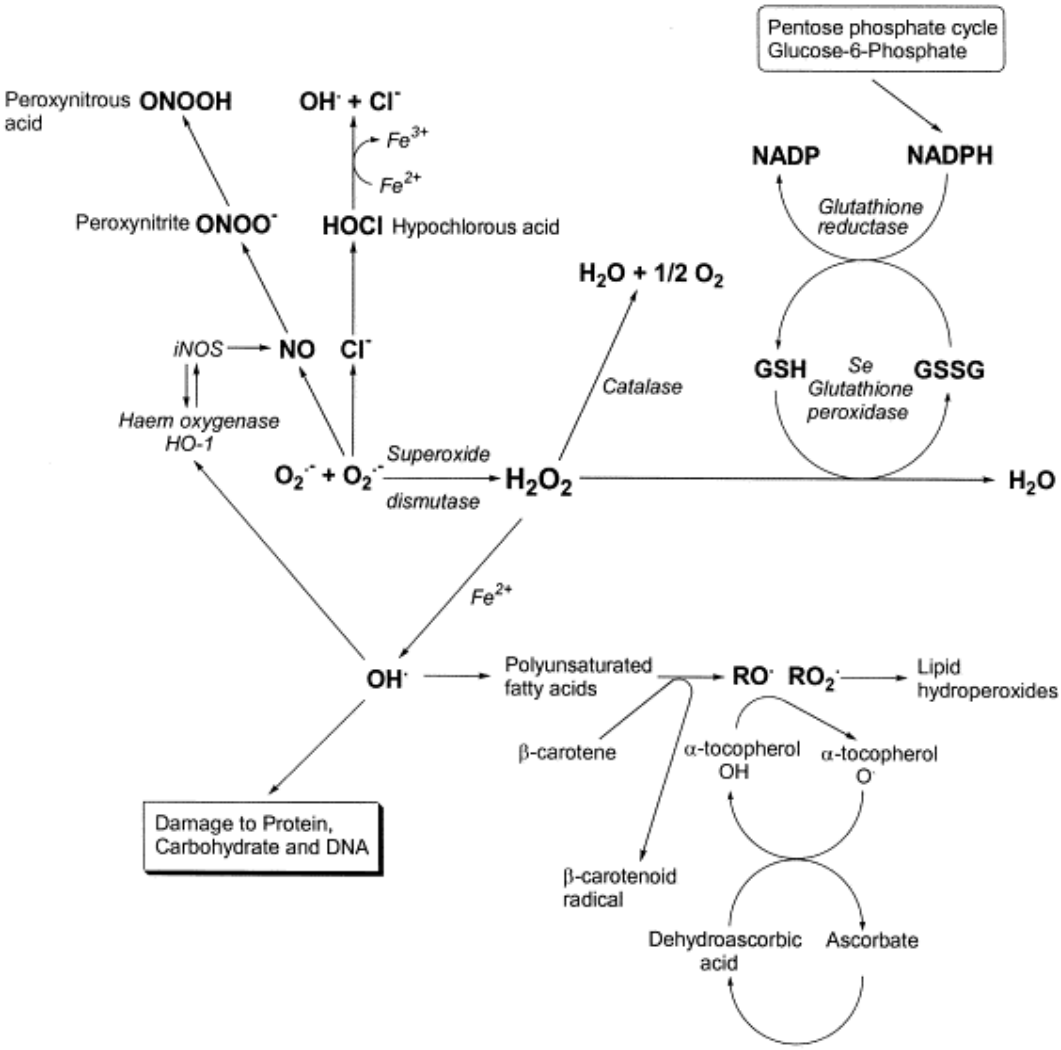

Figure 1.6: Cascade showing co-operation between enzyme and nonenzymatic antioxidants for scavenging ROS (Crichton, 2002).

1.2.5 Oxidative stress and the brain

1.2.5.1 Vulnerability of the brain to oxidative stress

The brain and the nervous system may be particularly vulnerable to free radical attack for a number of reasons (Halliwell and Gutteridge, 1985; LeBel and Bondy, 1991). Some of the most important reasons are summarized below.

1. Although the brain only makes up a small percentage of the total body weight, it consumes a relatively large amount of the O_2 inspired and carries out the turnover of large quantities of ATP at a high rate. Given that oxygen-derived radicals are toxic, it is not surprising that neural tissue may be destroyed at a more rapid rate than other organs (Reiter, 1998).
2. Neurons are non-replicating cells (Reiter 1998). Any damage to neurons by reactive oxygen species therefore tends to be cumulative over time (Cui *et al*., 2004).
3. The membrane lipids in the brain are rich in polyunsaturated fatty acid (PUFA) side chains, which are particularly susceptible to free radical attack. Free radical attack on PUFA side chains can initiate the process of lipid peroxidation and once underway the process is self-propagating (Sevanian and McLeod, 1997).
4. Despite the high oxygen utilization by the brain, it is not well equipped to combat oxidative attack. The brain has relatively low levels of important antioxidative enzymes (Savolainen, 1978; Bondy, 1997). The brain is poor in catalase activity and has only moderate amounts of superoxide dismutase (SOD) and glutathione peroxidase (Halliwell, 1992).
5. Several areas of the brain (e.g. the *globus pallidus* and *substantia nigra*) are rich in iron. Unbound iron can serve as a catalyst and can generate free radicals via either the Fenton reaction or the Haber-Weiss reaction (Sabrzadeh *et al*., 1987).
6. The cerebrospinal fluid (CSF) has no significant iron-binding capacity because it has low levels of the iron binding protein, transferrin (Halliwell, 1992). Iron plays an important role in the brain (Youdim, 1988; Halliwell, 1992), especially

with regard to learning and memory. It has also been suggested that iron ions are necessary for the correct binding of certain neurotransmitters to their receptors (Youdim, 1988). A high content of brain iron may therefore be essential, especially during development, however a consequence of this is that damage to brain cells may release catalytic iron ions that can stimulate free radical generation.

7. There is a high concentration of ascorbic acid in the grey and white matter of the brain. There are transport systems in the choroidal plexus and neural tissue, which serve to concentrate ascorbic acid into brain cells and the CSF (Cui *et al*,. 2004). In the absence of transition metal ions ascorbic acid has well documented antioxidant properties. However, mixtures of ascorbate with iron or copper generate free radicals. If catalytic iron were released as a result of injury to brain cells, endogenous ascorbate in the brain might then stimulate •OH formation in the brain and the CSF (Halliwell 1992).
8. The release of excitatory neurotransmitters, such as glutamate, induces a series of events in the postsynaptic neuron, leading to the generation of reactive oxygen species such as NO• (Reiter, 1998).
9. Nitric oxide synthase (NOS) is widespread in brain tissue (Hope *et al*., 1991). NOS is activated by calmodulin (Cui *et al*., 2004). The interaction between NO• and $O_2^{\bullet-}$ to generate the peroxynitrite anion ($ONOO^-$) has been suggested to be involved in not only the normal metabolism of the neuron but also in its degeneration (Cui *et al*., 2004).
10. Melatonin is an important endogenous antioxidant produced by the pineal gland. Melatonin is a good scavenger of ROS. However, the concentration of this protective neurohormone decreases with increasing age (Cui *et al*., 2004).
11. Dopaminergic neurons in the *substantia nigra pars compacta* (SNpc) are particularly vulnerable to oxidative stress as a result of the ROS generated during DA metabolism.
12. The blood brain barrier (BBB), although it prevents many toxins from gaining access to the CNS, it also restricts the entry of a number of antioxidants into the CNS (Reiter, 1998).

1.3 PARKINSON'S DISEASE

1.3.1 Introduction and clinical features

Parkinson's disease (PD), first described by James Parkinson in 1817 (Parkinson, 1817), is the second most common neurodegenerative disease after Alzeimer's disease, affecting approximately 2 % of the population after 65 years. The mean onset of the disease is around 60 years, with a mean duration of 13 years (Hughes *et al*., 1993). Less frequently, PD may have an onset below 40 years (Golbe, 1991). The cardinal clinical features of PD are resting tremor, rigidity, bradykinesia and a later loss of postural reflexes.

Pathologically, PD is characterized by a progressive loss of neuromelanin-containing DA neurons in the *pars compacta* of the *substantia nigra*, with intracellular proteinaceous inclusions named Lewy bodies (Blum *et al*., 2001) and a reduction in striatal DA content (Ehringer and Hornykiewicz, 1960). Other lesions, such as degeneration of the noradrenergic locus coeruleus (Greenfield and Bosanquet, 1953), the dopaminergic ventral tegmental area (Agid *et al*., 1990) and the ascending cholinergic from the Meynert basalis nucleus (Candy *et al*., 1983), were also observed.

A subclinical phase exists prior to the appearance of PD symptoms. During this phase, striatal compensatory mechanisms (Agid *et al*., 1990; Anglade *et al*., 1995), such as sensitization of dopaminergic receptors (Agid *et al*., 1990) and enhanced neuronal activity occur. PD is therefore not clinically obvious before at least 50 – 70 % of the dopaminergic neurons in the *substantia nigra pars compacta* (SNpc) are lost, leading to a greater than 80 % reduction in DA levels in the striatum (Deumens *et al*., 2002). For this reason the etiology of PD remains difficult to establish.

1.3.2 Causative factors in PD

Despite numerous attempts at elucidation, the etiology of PD remains unclear. It is hypothesized that the cause of neurodegeneration in PD is multi-factorial in terms of both etiology and pathogenesis.

One theory states that nigral neurons are sensitive to environmental contaminants that inhibit mitochondrial complex I and lead to impaired energy metabolism (Orth and Schapira, 2002). This theory arose from the discovery of a mitochondrial toxin, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) (Langston *et al*., 1983), which produces selective nigral neuronal death in human and animal models (Gerlach and Riederer, 1996) and induces the motor symptoms associated with PD (Langston *et al*., 1983). Figure 1.7 depicts a hypothetical mechanism by which MPTP exerts its neurotoxicity.

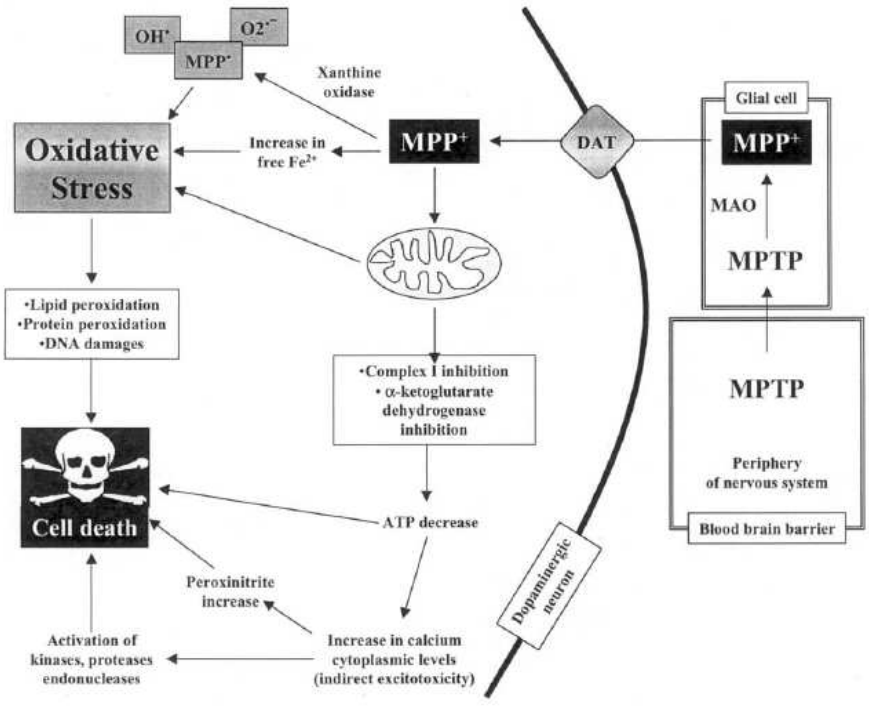

Figure 1.7: Hypothetical mechanism of MPTP toxicity. MPTP crosses the BBB and is transformed by glial monoamine oxidase (MAO) into MPP^+. MPP^+ leads to a major inhibition of the respiratory chain but also to oxidative stress, both triggering cell death (Blum *et al*., 2001).

However, although MPTP and analogues, such as tetrahydroisoquinolines (Niwa *et al*., 1987; Naoi *et al*., 1993) or other exogenous/endogenous compounds (Gerlach and Riederer, 1996), such as carbon monoxide (Ringel and Klawans, 1972; Choi and Cheon, 1999), β-carbolines (Collins and Neafsey, 1985) or rotenone (Betarbet *et al*., 2000), can produce dopaminergic lesions, none have been shown to be responsible for the majority of PD cases.

The proteolytic stress hypothesis ascribes the loss of nigral neurons in PD to the toxic accumulation of misfolded and aggregated proteins. Of particular relevance to PD is α-synuclein, a protein encoded by a gene mutated in a rare early onset, familial form of PD. Mutations in α-sunuclein and ubiquitin C-terminal hydrolase L1 (UCH-L1) have been linked to autosomal-dominant PD and mutations in parkin to autosomal-recessive PD. Parkin and UCH-L1 are key enzymes participating in the ubiquitin-proteasome system (UPS), which is responsible for the degradation of ubiquinated proteins (Lim *et al*., 2003). In addition to a defect in the UPS, postmortem nigral tissue from sporadic PD patients show a significant defect in the 20 S proteasome, the primary cellular machinery responsible for degrading non-ubiquinated proteins (Dunlop *et al*., 2002). Blocking or overwhelming the degradation of these proteins can lead to cell death (Chung *et al*., 2001). Contribution of genetic mutations cannot explain sporadic and late-onset cases of PD (Tanner *et al*., 1999).

Although the etiology of the degeneration in PD is still ill-defined, there is growing interest in the phenomena underlying the degeneration process. In particular, oxidative stress as a result of the highly oxidative intracellular environment within dopaminergic neurons has been put forward as one of the major causes of the nigral degeneration (Lotharius and Brundin, 2002). The normal enzymatic degradation of DA induces the formation of H_2O_2 via MAO activity. The nonenzymatic auto-oxidation of DA also produces H_2O_2 and the formation of neuromelanin that potentiates hydroxyl radical formation when it combines with iron (Fahn and Cohen, 1992; Jellinger *et al*., 1992; Jenner *et al*., 1992). Finally, there is a decrease in cells synthesizing glutathione peroxidase (Damier *et al*., 1993) in the vulnerable part of the *substantia nigra* (SN).

1.3.3 Evidence of oxidative stress in PD

Evidence of oxidative stress in PD is inferred by the following findings:

1. Carbonyl modifications, which are indicative of protein oxidation, are increased 2-fold in the SN of PD patients compared to the basal ganglia and prefrontal cortex of normal subjects (Floor and Wetzel, 1998).
2. The SN of Parkinsonian brains has a higher content of malondialdehyde (MDA) (Ilic *et al*., 1999) and 4-hydroxy-2-nonenal (HNE) (Yoritaka *et al*., 1996), adehydes generated during lipid peroxidation, compared to control brains.
3. Decreased levels of GSH localized to surviving neurons has been detected in the SN of PD patients compared to age-matched controls (Pearce *et al*., 1997; Sofic *et al*., 1992) Furthermore, there is a significant increase in the levels of GSSG (the oxidation product of GSH) in PD brains.
4. Mitochondria are damaged and oxidative phosphorylation is impaired (Ebadi *et al*., 2001; Shoffner *et al*., 1992).
5. Iron metabolism is impaired (Jellinger *et al*., 1990; Riederer *et al*., 1989; Youdim *et al*., 1993).
6. The levels of cysteinyl-catechols is increased in the SN during aging (Fornstedt *et al*., 1989, 1990a) and even more so in PD (Fornstedt *et al*., 1989). Protein crosslinking is also increased (Berlett and Stadman, 1997), signified by the presence of Lewy bodies (Leroy *et al*., 1998).
7. Levels of 8-hydroxyguanosine, a nucleoside oxidation product, is increased 16-fold in the SN of PD brains compared to controls (Sanchez-Ramos., 1994; Zhang *et al*., 1999).
8. Antibodies for catechol-modified proteins were found in the serum of PD patients (Rowe *et al*., 1998).
9. A reduction in the CSF levels of superoxide dismutase and glutathione reductase was found in *de novo* Parkinsonians (Ilic *et al*., 1999).

1.3.4 Dopamine and PD

1.3.4.1 DA synthesis, storage, release, reuptake and metabolism

Tyrosine is usually the starting point in the biosynthesis of DA (figure 1.8). Tyrosine is obtained from the diet and can also be synthesized from dietary phenylalanine by phenylalanine hydroxylase in the liver or by tyrosine hydroxylase in the DA neuron. Tyrosine in the circulation is taken up into the brain via a low-affinity amino acid transport system and then enters the dopaminergic neurons by high- and low-affinity amino acid transporters. The conversion of tyrosine to L-Dopa (LD), by tyrosine hydroxylase is the rate limiting step in the biosynthesis of DA. Aromatic amino acid decarboxylase (AADC) is then responsible for the conversion of LD to DA (Elsworth and Roth, 1997)

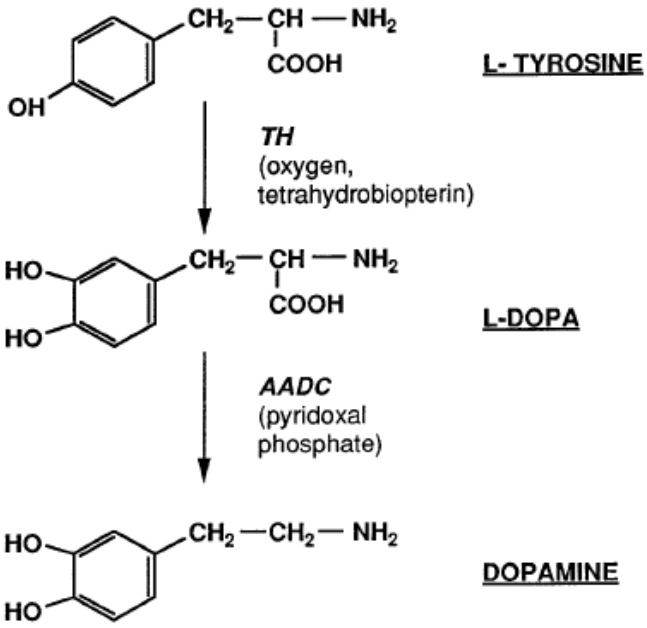

Figure 1.8: Biosynthesis of DA. Enzymes involved are tyrosine hydroxylase and aromatic amino acid decarboxylase (AADC). Cofactors for these enzymes are given in parenthesis (Elsworth and Roth, 1997).

In dopaminergic neurons, intracellular DA (0.1 – 1 mM) (Jonsson, 1971) is efficiently incorporated into vesicles for neuronal transmission. The arrival of an action potential in the dopaminergic neuron results in a change in membrane protein conformation and the subsequent influx of calcium ions. This is a key part of the stimulus responsible for the fusion of vesicles with the neuronal membrane. By the process of exocytosis, DA vesicles discharge their soluble contents into the synapse (Kelly, 1993). The extent of DA release is dependent on the rate and pattern of neuronal firing (Grace and Bunney, 1995).

In the synaptic cleft DA interacts with autoreceptors and postsynaptic receptors. Autoreceptors are present on most parts of the dopaminergic neurons and are responsive to both dendritic and terminal DA release. Stimulation of autoreceptors in the somatodendritic region decreases the firing rate of the dopamine neurons and stimulation of autoreceptors located on the DA nerve terminals inhibits the synthesis and/or release of DA. DA also interacts with postsynaptic D_1 and D_2 receptors. When stimulated, the D_1 receptors generally enhance adenylate cyclase activity and the D_2 receptors inhibit adenylate cyclase activity (Elsworth and Roth, 1997).

DA action at the synapse is terminated predominantly by re-uptake into the presynaptic terminal through the DA transporter, DAT. It has been estimated that DA can be concentrated 100- to 1000-fold by the transporter. Once back in the DA terminals, the neurotransmitter can be repackaged into vesicles (Elsworth and Roth, 1997).

The main enzymes responsible for the metabolism of DA are shown in figure 1.9. The principle locations of these enzymes are shown in table 1.1.

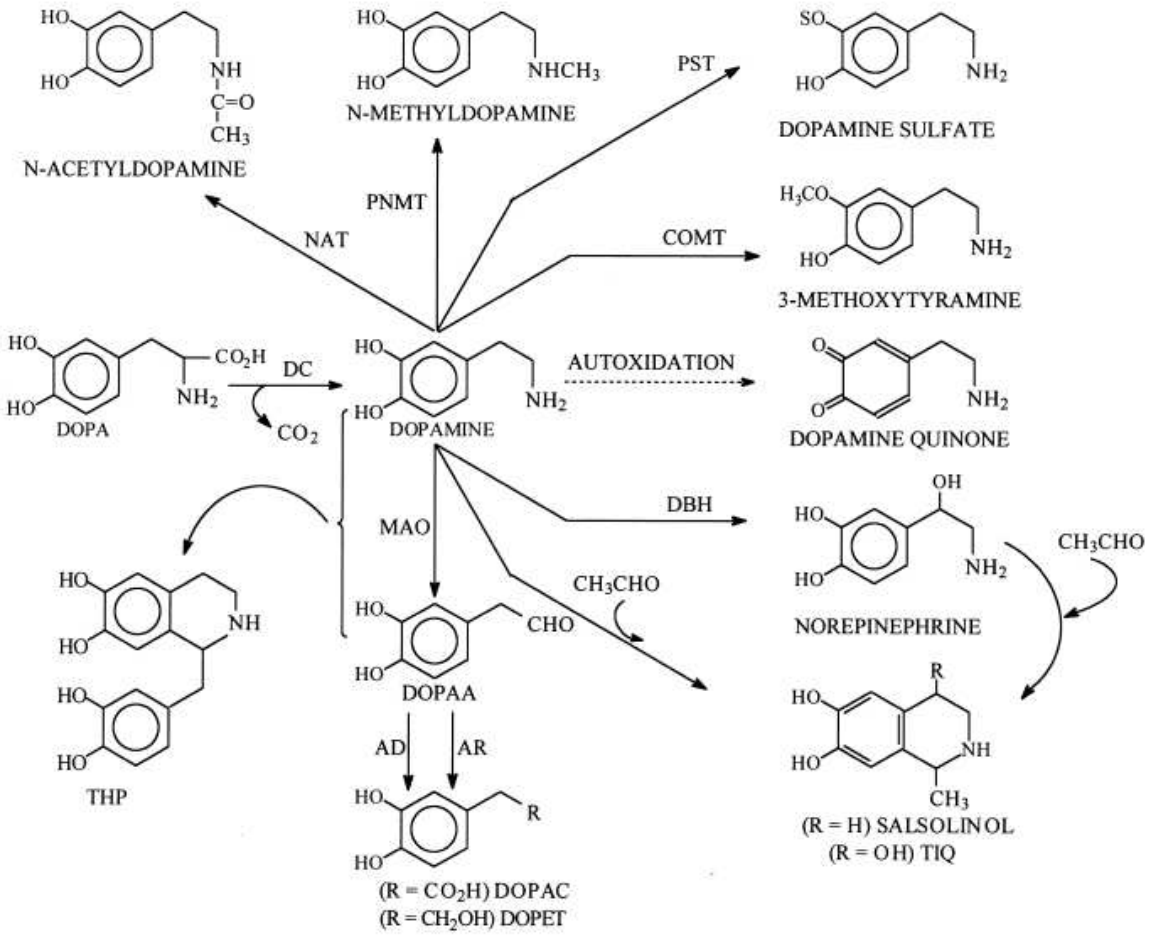

Figure 1.9: Main pathways of DA metabolism. Enzymes involved are monoamine oxidase (MAO), catechol-O-methyltransferase (COMT), phenolsulfotransferase (PST), dopamine β-hydoxylase (DBH), N-acetyltransferase (NAT) and phenylethanolamine N-methyltrasferase (PNMT) (Nappi and Vass, 1998).

The relative abundance and activity of the enzymes vary according to cell type, brain region, and species being examined. These factors determine the concentration of a particular metabolite present under a given set of conditions. MAO and COMT represent the two main catabolic enzymes affecting dopaminergic neurotransmission. The major end product of DA metabolism in primate brain is unconjugated homovanillic acid (HVA), whereas in rat brain it is 3,4-dihydroxyphenylacetic acid (DOPAC), and a

significant amount of the DOPAC in the rat brain is sulfate conjugated (Elsworth and Roth, 1997). Rat and human phenolsulfotransferases (PST) have different specificities, with DOPAC having a higher affinity for rat brain PST than human brain PST (Roth *et al*., 1996). The major non-enzymatic route of DA metabolism involves its auto-oxidation to toxic quinone and semiquinone species with the concomitant generation of ROS.

Table 1.1: Principle locations of DA metabolizing enzymes in the striatum (Elsworth and Roth, 1997)

Enzyme	Subtype	Cellular location	Subcellular location
MAO	A	Dopaminergic and norepinephrine neurons	Mitochondria
MAO	B	Glia and serotonin neurons	Mitochondria
COMT	high Km	Glia	Soluble
COMT	low Km	Postsynaptic to dopamine neuron	Membrane-bound
PST	M	Postsynaptic to dopamine neuron	Cytosolic

1.3.4.2 Neurotoxicity of DA

DA has been implicated as an important vulnerability factor in PD because it has the potential to induce oxidative stress, proteolytic dysfunction and mitochondrial defects in nigral neurons (figure 1.10).

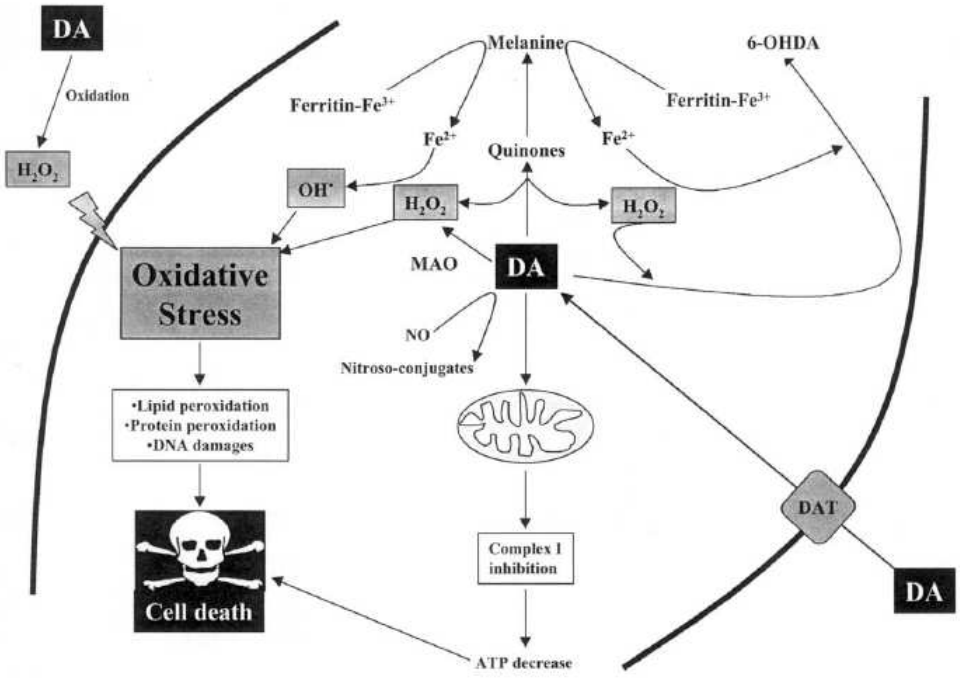

Figure 1.10: Hypothetical mechanism of DA toxicity. DA could induce dopaminergic neuronal death by increased ROS production, direct inhibition of the mitochondrial respiratory chain, and protein oxidation which may lead to proteolytic dysfunction and protein aggregation (Blum *et al*., 2001).

1.3.4.2.1 DA-dependent oxidative stress

One possible endogenous source of free radicals in the degenerative processes underlying PD may involve the MAO-mediated metabolism of DA and the auto-oxidation of DA. MAO activity could contribute to the neurotoxicity of DA by producing H_2O_2 and 3,4-dihydroxyphenylacetaldehyde (DOPALD) (figure 1.11), which are toxic to

catecholaminergic neurons (Mattammal *et al*., 1995). The intracellular auto-oxidation of DA generates H_2O_2 and DA quinone (DA-Q) (figure 1.11) (Graham *et al*., 1978; Sulzer and Zecca, 2000). H_2O_2, produced during the metabolism and auto-oxidation of DA can be converted to •OH by the Fenton reaction in the presence of ferrous iron (Fe^{2+}). Hydroxyl radicals are highly reactive and are capable of reacting with virtually every cellular macromolecule including proteins, lipids and DNA.

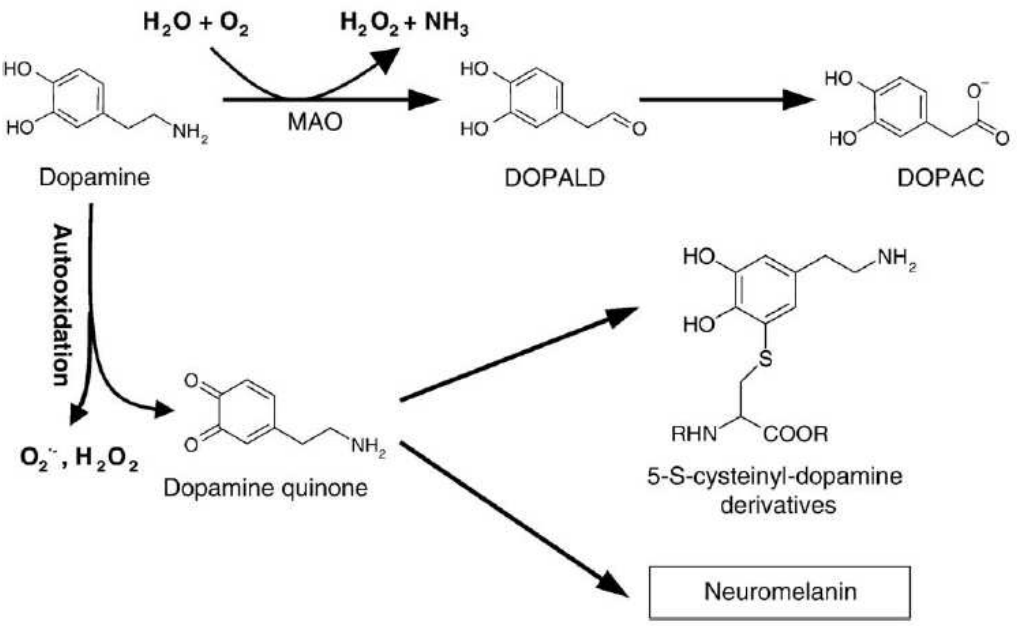

Figure 1.11: Oxidation of DA by MAO or by auto-oxidation leads to the production of H_2O_2, which can be converted to •OH and lead to the oxidation of proteins, lipids and nucleosides. Auto-oxidation of DA also results in the formation of DA-Q, which may covalently modify proteins or be converted to neuromelanin (Hald and Lotharius, 2005).

The electronegative nature of the ortho-oxygens on the catechol ring of DA-Q, and the electron-deficient and unstable aromatic ring confer a positive charge to positions 2 and 5 of the ring. This facilitates nucleophilic attack by sulfhydryls (Kato *et al*., 1986; Monks *et al*., 1992), which are present largely in GSH, cysteine or in proteins. DA-Q binding to cysteinyl groups in proteins leads to a loss of protein function (Hastings and Zigmond, 1994; Stadtman, 1992), while binding to GSH leads to the cellular depletion of GSH, resulting in an inadequate cytoplasmic antioxidant load. DA-Q has been shown to inhibit

glutamate and DA transporter function in synaptosomes (Berman and Hastings, 1997), inhibit tyrosine hydroxylase (TH) in cell free systems (Kuhn *et al*., 1999), and promote H^+ leakage from mitochondria resulting in uncoupling of respiration to ATP synthesis (Berman and Hastings, 1999; Khan *et al*., 2001).

DA-Q can further undergo internal rearrangement in which the aminoalkyl chain cyclizes, forming aminochrome. This reaction can be catalysed by oxygen (Graham, 1978; Senoh *et al*., 1959a), Fe^{2+}, cytochrome P450 (Segura-Aguilar, 1996), prostaglandin H synthase (COX-1 and -2) (Hastings, 1995), lactoperoxidase (Segura-Aguilar *et al*., 1998), xanthine oxidase (Foppoli *et al*., 1997), tyrosinase (Korytowski *et al*., 1987), $ONOO^-$, NO• (LaVoie and Hastings, 1997, 1999) and perhaps even $O_2^{\bullet-}$ and •OH (Ito and Fujita, 1982; Nappi *et al*., 1995; Spencer *et al*., 1995). The one-electron reduction of aminochrome has been proposed to be one of the major sources of endogenous generation of ROS involved in the degenerative process leading to PD (figure 1.12) (Baez *et al*., 1995; Paris *et al*., 2001; Segura-Aguilar *et al*., 1998, 2001).

The one-electron reduction of aminochrome induces neurotoxicity by generating leukoaminochrome o-semiquinone, an endogenous neurotoxin (equation 1.11).

Aminochrome + NADPH → Leukoaminochrome-o-SQ• (Equation 1.11)

The toxicity of leukoaminochrome-o-SQ• depends on its high reactivity with oxygen (equation 1.12), which generates a redox cycling with concomitant formation of intracellular ROS and the depletion of NADPH (figure 1.12, reactions 3 and 4)

Leukoaminochrome-o-SQ• + O_2 → Aminochrome + $O_2^{\bullet-}$ (Equation 1.12)

The preceding two reactions tend to continue until NADPH is fully consumed, or until O_2 is depleted (Segura-Aguilar *et al*., 1998). The $O_2^{\bullet-}$ formed during this redox cycling is converted to H_2O_2 by SOD. H_2O_2 can then generate the extremely toxic •OH via the Fenton reaction of Haber-Weiss reaction.

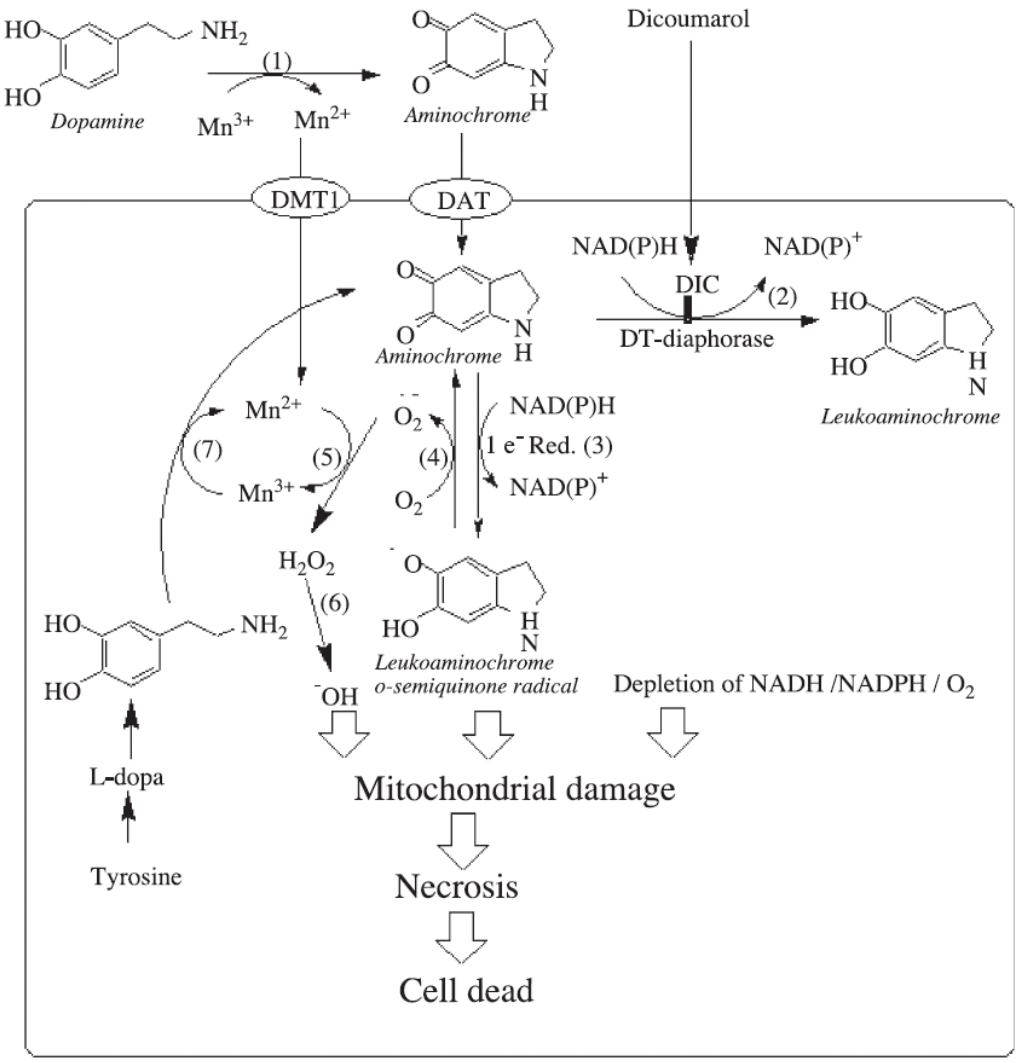

Figure 1.12: Possible mechanism of toxicity involved in the one-electron reduction of aminochrome (Arriagada *et al*., 2004).

1.3.4.2.2 Linking DA to mitochondrial dysfunction

The reduced complex I function associated with PD (Schapira, 1994; Sherer *et al*., 2002) may be dependent on DA. As mitochondrial proteins are rich in sulfhydryl moieties, it is not surprising that oxidized catechols, such as DA inhibit mitochondrial complex I (Ben-Shachar *et al*., 1995; Morikawa *et al*., 1996; Przedborski *et al*., 1993), promote opening

of the mitochondrial permeability pore, uncouple oxidative phosphorylation and result in mitochondrial swelling (Berman and Hastings, 1999). DA has been shown to inhibit complex I when injected into the brain ventricles of rats (Ben-Shachar *et al*., 1995). This toxic effect may be due to H_2O_2 formed during DA metabolism or by 7-(2-aminoethyl)-3,4-dihydro-5-hydroxy-2H-1,4-benzothiazine-3-carboxylic acid (DHBT-1), an oxidation byproduct of 5-S-cysteinyl-dopamine, which has been shown to inhibit mitochondrial complex I in isolated mitochondria (Li and Dryhurst, 1997). In addition to this, rotenone only exerts its toxicity in dopaminergic neurons although it inhibits complex I throughout the brain. This suggests that dopaminergic neurons have an intrinsic sensitivity to complex I defects (Sherer *et al*., 2002).

1.3.4.2.3 Linking DA to defective proteolysis

Sulfhydryls tend to bind covalently to the catechol ring of DA at positions 2 and 5 (equation 1.13) (Ito *et al*., 1988; Kato *et al*., 1986).

DA-Q + cysteine → 2-cysteinyl-DA [1 part] + 5-cysteinyl-DA [6 parts] + 2,5-dicysteinyl-DA [minor] (Equation 1.13)

Cysteinyl-DA is more-readily oxidized to the o-quinoidal form than the parent catechol (Monks *et al*., 1992; Shen *et al*., 1996). This process accounts for crosslinking in protein, with additional loss of function and eventual appearance of protein aggregates such as Lewy bodies in Parkinson's disease (Tran and Miller, 1999).

DA has been shown to form covalent adducts with α-synuclein. This synaptic vesicle protein of unknown function has a greater tendency to aggregate when oxidized (Giasson *et al*., 2000). The formation of covalent, oxidative adducts between DA and α-synuclein promotes the retention of α-synuclein as a protofibrillar species (Conway *et al*., 2001). Protofibrillar α-synuclein is, in turn, capable of permeabilizing synthetic vesicles (Volles *et al*., 2001) potentially leading to a leakage of DA from synaptic storage sites. Mutation in α-synuclein, which also increases protofibril formation (Conway *et al*., 2000), could

therefore result in increased cytoplasmic levels of DA and DA-dependent oxidative stress in some familial forms of PD. Studies using cultured human dopaminergic neurons suggest that mutant α-synuclein triggers an elevation of cytoplasmic DA, oxidative stress and DA-dependent toxicity (Lotharius *et al*., 2002; Xu *et al*., 2002). DA-dependent oxidation of intracellular targets like α-synuclein, which could be important for neurotransmitter storage, may explain the selective vulnerability of dopaminergic neurons in PD (Lotharius and Brundin, 2002).

1.3.5 Iron and PD

A major role for iron in the pathogenesis of PD is supported by several findings:

1. Levels of iron are increased in PD brains compared to age-matched control brains.
2. The region of iron accumulation co-localizes with the region of degeneration, i.e. the SN. Iron levels in the SN are increased by approximately 35 % in PD brains compared to age-matched control brains (Dexter *et al*., 1989a; Sofic *et al*., 1988).
3. Iron has the ability to enhance the production of •OH and other ROS resulting in oxidative stress, the most likely cause of dopaminergic cell death in PD.
4. Iron has been demonstrated to interact with α-synuclein, resulting in its aggregation and suggesting a role for iron in Lewy body formation, a major pathological hallmark of PD (Kaur and Andersen, 2002).

Addtionally, what gives further credence to the role of iron in the pathogenesis of PD is the protection afforded by iron chelators in various paradigms of the disease.

1.3.5.1 The iron paradox

The unique physicochemical properties of iron make it an essential component in an enormous array of biological processes. Depending on the environment, it is capable of not only varying its oxidation state but also its electron spin and redox potential (Kaur

and Andersen, 2002). Iron therefore has an enormous capacity to serve multiple roles in vital biochemical reactions. However, if not appropriately shielded, ferrous iron can participate in one-electron transfer reactions leading to the production of extremely toxic free radicals, particularly via the Haber-Weiss and Fenton reactions (Comporti, 2002). Intracellualr iron levels are therefore stringently regulated as a labile iron pool (LIP) which provides enough iron for vital iron-dependent biochemical reactions but limits the availability of iron to participate in free-radical generating chemistry.

An example of this "iron paradox" exists within the dopaminergic neurons of the SN. The enzyme tyrosine hydroxylase, which synthesizes L-Dopa (LD) from tyrosine requires Fe^{2+} as an essential co-factor. The conversion of tyrosine to LD is normally the rate limiting step in the biosynthesis of DA (Elsworth and Roth, 1997). However, iron also promotes the auto-oxidation of DA in SN neurons, releasing H_2O_2 in the process (Ben-Shachar *et al*., 1995), the precursor to the highly reactive and toxic •OH. Iron also catalyses the conversion of excess DA to neuromelanin, an insoluble black-brown pigment that accumulates in all dopaminergic neurons with age in humans (Sulzer *et al*., 2000). Neuromelanin is usually neuroprotective and sequesters redox active metals in the cell with a high affinity for Fe^{3+}. However, when bound to excess Fe^{3+}, neuromelanin tends to become a pro-oxidant reducing Fe^{3+} back to Fe^{2+}, which is then released due to weak affinity (Ben-Shachar *et al*., 1991). This increases the LIP and the fraction of iron available for reacting with H_2O_2 to produce •OH. Dopaminergic neurons must therefore regulate iron levels very stringently to keep the generation of •OH under check without compromising DA synthesis.

1.3.5.2 Sources of iron for free-radical generating chemistry

In mammals, iron is absorbed from the gut and originates from food and beverages we consume that are fortified with iron (figure 1.13).

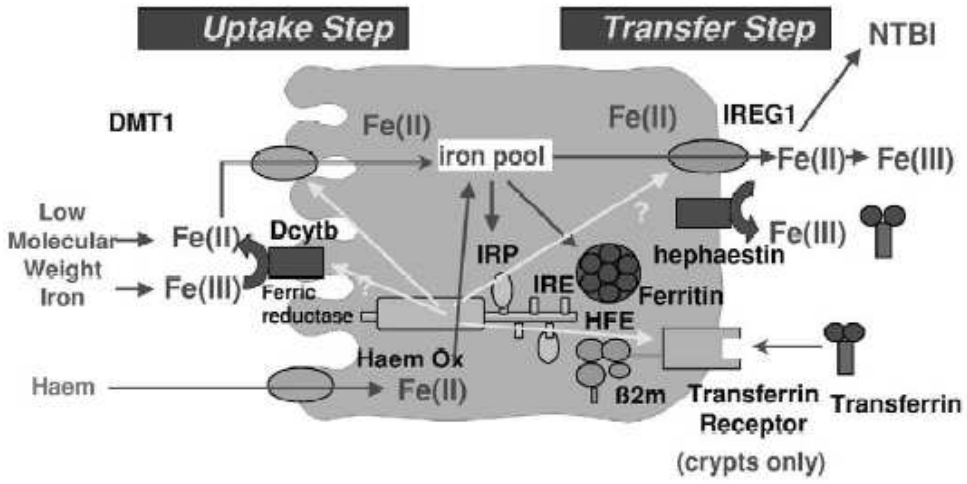

Figure 1.13: Schematic representation of iron absorption in normal subjects (Crichton *et al*., 2002).

Iron is taken up from the gastrointestinal tract (GIT) as either heme iron or nonheme iron. The uptake of heme iron across the apical membrane of the intestinal mucosal cell involves a specific heme receptor on the apical membrane of the cell (Grasbeck *et al*., 1982). Its iron is then released by the action of heme oxygenase as Fe^{2+}, with concomitant production of porphobilinogen and CO (Beale and Yeh, 1999). Nonheme dietary iron seems to cross the brush border membrane, after reduction by duodenal ferric reductase, Dcytb (McKie *et al*., 2001). The Fe^{2+} is then transported into the intestinal cell by the divalent metal transporter 1 (DMT-1). Within the intestinal cell, Fe^{2+} derived from both heme and nonheme iron enters a low molecular weight iron pool. This iron can either be stored in ferritin within the mucosal cell or be transported to the basolateral membrane. The diffusion of Fe^{2+} across the basolateral membrane is facilitated by IREG1, a transmembrane iron transporter protein (McKie *et al*., 2000). Hephaestin, a membrane bound protein facilitates the oxidation of Fe^{2+} to Fe^{3+} in order to allow rapid binding of iron to tranferrin in the plasma (Vulpe *et al*., 1999).

Transferrin delivers iron to cells expressing transferrin receptors (figure 1.14.). The bilobal transferrin molecule binds 2 moles of Fe^{3+} per mole of protein with very high affinity at pH 7.4. Diferric transferrin binds to its receptor at the cell surface and the

transferrin-transferrin-receptor complex is then internalized in clathrin-coated vesicles. The vesicles then lose their coat and fuse with endosomes. The interior of the endosomal compartment is maintained at pH 5.5 which facilitates the release of iron, as Fe^{3+} from the transferrin-transferrin-receptor complex. The Fe^{3+} is then reduced to Fe^{2+} prior to its transport out of the endosome by DMT-1. The unloaded transferrin is ejected from the cell and the Fe^{2+} released from it can be used for the synthesis of intracellular iron proteins. Excess iron is stored in the protein ferritin (Crichton *et al*., 2002).

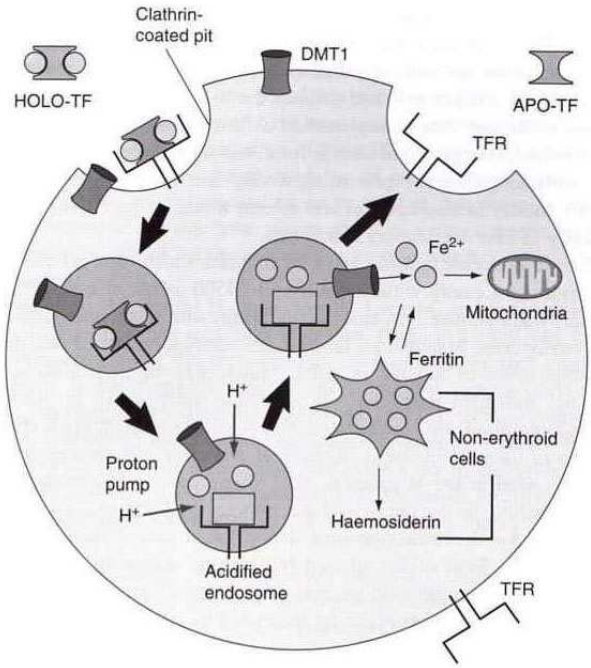

Figure 1.14: The transferrin cycle. Delivery of iron to cells by transferrin (Zecca and Youdim, 2004).

Transferrin is resistant to damage by $O_2^{\bullet-}$, H_2O_2 and other oxidants, and the iron bound to this protein will not catalyze free radical reactions (Aruoma and Halliwell, 1987). By contrast, some iron can be reductively mobilized from ferritin by $O_2^{\bullet-}$ and by radicals generation during lipid peroxidation (Biemond *et al*., 1986; Bolann and Ulvik, 1990). Iron catalytic for Fenton chemistry appears to be available within cells, both as a labile iron pool and as iron releasable from ferritin.

1.3.5.3 Iron and protein aggregation

One of the primary neuropathological criteria for a confirmed postmortem diagnosis of PD is the presence of intracytoplasmic filamentous inclusions known as Lewy bodies and Lewy neurites in some surviving dopaminergic nigral neurons (Kaur and Andersen, 2002). The major fibrillar material of these inclusions is α-synuclein. Mutations in the gene for α-synuclein cause a form of familial PD, suggesting that alterations in α-synuclein may be involved in the pathogenesis of the disease. Hashimoto *et al* (1999) demonstrated that iron-induced oxidative stress can result in aggregation of α-synuclein *in vitro*. Further studies by Osterova-Golts *et al*. (2000) carried out in neuroblastoma cells over-expressing either wild type or mutant α-synuclein demonstrated that iron in combination with other free radical generators stimulates the formation of intracellular aggregates containing both α-synuclein and ubiquitin. A study by Münch *et al*. (2000), demonstrated that advanced glycation end-products, markers of iron-induced oxidative stress, were found to promote crosslinking of α-synuclein in the brains of patients with incidental Lewy body disease, generally viewed as being pre-Parkinsonian patients. Golts *et al*. (2002) demonstrated the presence of iron-induced alterations in the fluorescence of the four-tyrosine residues of α-synuclein, suggestive of iron-related conformational changes which could eventually lead to protein aggregation. These studies link iron to a major pathological hallmark in PD and gives further credence to the role of iron in the pathogenesis of PD.

1.3.5.4 Iron and endogenous neurotoxin formation

Researchers have hypothesized that under conditions of chronic oxidative stress, a diversion of DA metabolism towards aberrant oxidative routes may be induced. A new mechanism of DA toxicity has been proposed based on this concept. Pezzella *et al*. (1997) reported that products of lipid peroxidation, that are increased in PD brain, can convert DA to 6-hydroxydopamine (6-OHDA), a potent neurotoxin. 6-OHDA is able to elicit selective destruction of peripheral and central catecholinergic neurons via

spontaneous oxidative conversion to potentially toxic dopaminergic quinone species. Oxidation of DA in the presence of ferrous iron demonstrated that this neurotransmitter can also be converted to 6-OHDA by •OH *in vitro*. It has been postulated that DA may also be converted to 6-OHDA *in vivo*, perhaps amplifying the neurodegenerative events that eventually lead to dopaminergic cell death (Napolitano *et al*., 1999).

1.3.6 Neurotoxicity of 6-OHDA

6-Hydroxydopamine is a common neurotoxin used to experimentally model nigral degeneration *in vitro* as well as *in vivo*. It was originally isolated by Senoh in 1959 (Senoh and Witkop, 1959a,b; Senoh *et al*., 1959a,b). Its biological effects were first reported by Porter *et al*. (1963, 1965) and Stone *et al*. (1963), who demonstrated that 6-OHDA induces norepinephrine (NE) depletion in the autonomic nervous system of the heart. Shortly thereafter, several studies demonstrated the ability of 6-OHDA to destroy sympathetic nerve terminals (Thoenen *et al*., 1967; Tranzer and Thoenen, 1967, 1968; Thoenen and Tranzer, 1968).

6-OHDA is unable to cross the BBB and destruction of central catecholamine neurons by this neurotoxin can only be achieved by direct intracerebral administration. In experimental models of PD, 6-OHDA is preferentially injected into the striatum, SN or the ascending medial forebrain bundle, destroying nigral dopaminergic neurons and depleting striatal DA, thus reproducing the physiopathological features responsible for motor impairments in PD (Blum *et al*., 2001)

Some evidence exists that 6-OHDA may be a physiological endogenous neurotoxin. Several studies demonstrated the presence of 6-OHDA in both rat (Senoh and Witkop, 1959a,b; Senoh *et al*., 1959a,b) and human brain (Curtius *et al*., 1974) as well as in the urine of patients with PD (Andrew *et al*., 1993). Nigral dopaminergic neurons contain significant amounts of DA, iron and H_2O_2. A non-enzymatic reaction between these elements may generate 6-OHDA *in vivo* (Slivka and Cohen, 1985; Jellinger *et al*., 1995; Linert *et al*., 1996).

The ability of 6-OHDA to mimic PD is reinforced by the fact that ROS generation and mitochondrial defects are induced by this neurotoxin (figure 1.15).

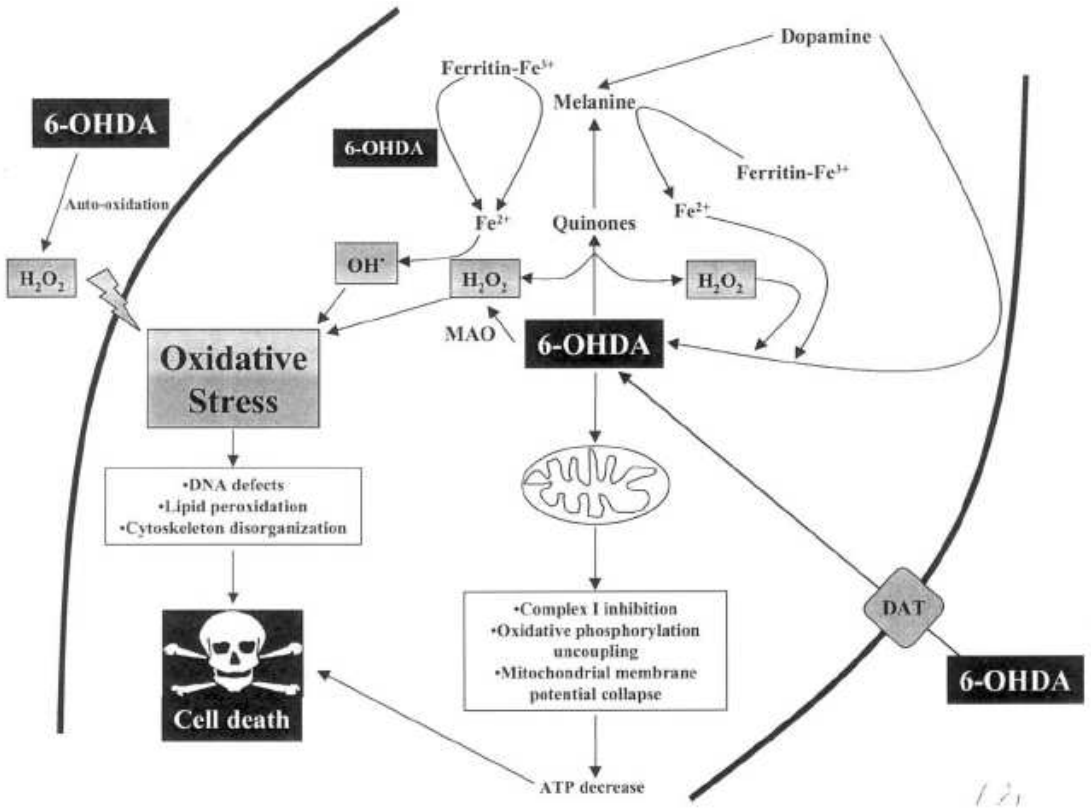

Figure 1.15: Hypothetical mechanism of 6-OHDA toxicity (Blum *et al*., 1991).

Similiarly to DA, 6-OHDA could induce catecholaminergic cell death by the generation of ROS during intra- and extracellular auto-oxidation, H_2O_2 formation induced by MAO activity or via direct inhibition of the mitochondrial respiratory chain.

Studies by Glinka and Youdim (1995) and Glinka *et al*. (1996, 1998) suggest that 6-OHDA directly targets the mitochondrial respiratory chain and inhibits complex I in isolated brain mitochondria. This could result in a depletion of ATP production by the mitochondria and subsequent cell death.

6-OHDA, like DA is a substrate for MAO (Breese and Traylor, 1971; Karoum *et al*., 1993). The enzymatic degradation of 6-OHDA by MAO therefore gives rise to H_2O_2. In

addition to this, under physiological conditions, 6-OHDA undergoes a very rapid and nonenzymatic auto-oxidation (Heikkila and Cohen, 1972; Seitz *et al*., 2000; Soto-Otero *et al*., 2000) which generates several toxic species including quinones (Saner and Thoenen, 1971), $O_2^{\bullet-}$, H_2O_2 and •OH (Cohen and Heikkila, 1974). The greater cytotoxicity of 6-OHDA compared to other catecholamines, including DA, is directly related to its higher rate of auto-oxidation compared to these compounds.

The formation of ROS by 6-OHDA may also be amplified by iron. Indeed, iron levels are increased in the striatum and SNpc following 6-OHDA injection (Hall *et al*., 1992; He *et al*., 1996; Oestreicher *et al*., 1994). The contribution of iron to the neurotoxicity of 6-OHDA is also suggested by studies showing that iron chelators are able to prevent some of the deleterious effects of 6-OHDA (Borisenko *et al*., 2000). Studies have shown that 6-OHDA is able to reduce and release iron, as Fe^{2+} from the main intracellular iron transport protein, ferritin (Jameson, 2004). This increases the size of the LIP and also the quantity of iron available for the generation of ROS via Fenton chemistry.

The 6-OHDA-induced oxidative stress reduces cellular antioxidant capabilities (Kumar *et al*., 1995), impairs intracellular redox potential regulation (Shiraga *et al*., 1993), causes lipid peroxidation as demonstrated by an increase in MDA (Kumar *et al*., 1995) and results in oxidative damage to DNA (Bruchelt *et al*., 1991; Gee *et al*., 1992).

1.3.7 Treatment of PD

1.3.7.1 Levodopa (LD)

1.3.7.1.1 Introduction

In 1960, Ehringer and Hornykiewicz analysed 31 fresh brains from neurologically normal adults, Huntington's disease, postencephalitic parkinsonism, extrapyramidal symptoms of unknown etiology, and idiopathic PD, and concluded that a significant striatal DA deficiency (approximate 90 % loss of striatal DA) was specific for Parkinson syndrome Ehringer and Hornykiewicz, 1960). Based on this observation, one year later Birkmayer and Hornykiewicz confirmed their earlier prediction, reporting that intravenous LD, the immediate precursor of DA, resulted in dramatic improvement of PD symptoms in 20 PD patients (Birkmayer and Hornykiewicz, 1961). LD thus became the first scientifically designed drug for the symptomatic treatment of a chronic, progressive, neurodegenerative disease (Rajput, 2001).

1.3.7.1.2 Pharmacology

1.3.7.1.2.1 Indications

LD is indicated for the treatment of PD, but not for drug-induced parkinson-like symptoms (Gibbon, 2003).

1.3.7.1.2.2 Mechanism of action

LD is converted by neuronal aromatic L-amino acid decarboxylase (AADC) into DA, hence restoring DA levels in the striatum and improving the symptoms of PD (Cannazza *et al*., 2005). However, the effectiveness of LD declines with time (Rajput, 2001). LD is usually administered with a peripheral decarboxylase inhibitor, e.g. benserazide or carbidopa to prevent the peripheral conversion of LD to DA.

1.3.7.1.2.3 Adult dosing

Oral treatment with Madopa® is 25 mg(benserazide)/100 mg(LD) eight hourly, increased by 25/100 mg weekly until the desired response is obtained. Usual effective range is 100/400 or 200/800 mg/day (Gibbon, 2003).

Alternatively the oral treatment with Sinemet® is 25 mg(carbidopa)/100 mg(LD) eight hourly, increased by 25/100 mg every day or alternate day until the desired response is obtained or a daily maximum of 200/800 mg/day is reached. Alternatively, the patient may take 12.5 mg(carbidopa)/100 mg(LD) once or twice daily, increased gradually by 12.5/125 mg every day or alternate day until the desired response is obtained or a maximum of 200/2000 mg/day is reached (Gibbon, 2003).

1.3.7.1.2.4 Drug interactions

Pharmacologic doses of pyridoxine enhance the extracerebral metabolism of LD and may therefore affect its therapeutic effect. LD should not be given to patients taking monoamine oxidase inhibitors (excluding selegiline) or within 2 weeks of their discontinuance, because such a combination can lead to a hypertensive crisis (Aminoff, 2004). Antipsychotic drugs such as phenothiazines and other antidopaminergic antipsychotic agents should be avoided. The likelihood of orthostatic hypotension is

increased when LD is administered with antihypertensive agents, such as methyldopa, particularly in the elderly (Gibbon, 2003).

1.3.7.1.2.5 Adverse effects

Response fluctuations become frequent with long-term LD treatment (Rajput, 1984; Cedarbaum, 1991; Chase *et al*., 1993). The most common motor response fluctuations are characterized by a predictable worsening at the end of a LD dose, known as wearing off, and an unpredictable short-duration worsening unrelated to the LD dose, known as "on-off" phenomenon (Chase *et al*., 1993; Rajput, 2001). The most common late motor adverse effect associated with long-term LD treatment is dyskinesia (Cedarbaum, 1991; Rajput, 2001). The pathophysiology of these motor complications has not as yet been elucidated. Other common adverse effects are gastrointestinal (GIT) symptoms, such as nausea and vomiting (in about 80 % of patients), anorexia, peptic ulceration and GIT bleeding and psychiatric disturbances, such as nervousness and anxiety (Gibbon, 2003).

1.3.7.1.2.6 Pharmacokinetics

Levodopa is rapidly absorbed from the small intestine, but its absorption depends on the rate of gastric emptying and the pH of the gastric contents. Food, especially protein will delay the appearance of LD in the plasma. Plasma concentrations usually peak 1-2 hours following an oral dose and the plasma half-life is usually 1-3 hours. Unfortunately, only about 1-3 % of the LD actually enters the brain unaltered, the rest being metabolized extracerebrally. LD is metabolized both centrally and peripherally (in the liver, kidneys, plasma and intestinal wall) to DA. The bioavailability of LD is enhanced by a peripheral decarboxylase inhibitor, such as benserazide and carbidopa, which inhibits the peripheral metabolism of LD to DA. LD is excreted in the urine mainly as DOPAC and HVA (Gibbon, 2003; Aminoff, 2004).

1.3.7.1.3 Neuroprotective potential of LD

The ease at which catechol containing compounds, such as LD and DA are oxidized, immediately brings to mind the potential for these compounds to act as antioxidants. By becoming oxidized, in place of some other important biomolecule, catechols can be viewed as cell protectors. Several studies have shown that LD and DA have an inhibitory effect on lipid peroxidation induced under various experimental conditions in brain homogenates (Zaleska and Floyd, 1985; Dostert *et al*., 1991; Yen and Hsieh, 1997), liposomes (Spencer *et al*., 1996), and linoleic acid (Liu and Mori, 1993). In rats with a unilateral 6-OHDA lesion, LD did not increase •OH content of the nigrostriatal dopaminergic system (Camp *et al*., 2000). Catechols are excellent free radical scavengers, however it should be noted that during this scavenging activity, the catechol compound is converted to oxidized products, i.e. semiquinone radicals and quinones. These products may also be toxic to other biomolecules, such as proteins (Ito *et al*., 1988; Boots *et al*., 2002). LD and DA therefore have both neuroprotective potential and toxic potential.

1.3.7.1.4 Neurotoxic potential of LD

Exogenous LD, although primarily metabolized to DA also undergoes auto-oxidation (Basma *et al*., 1995). There is increased DA turnover in the surviving dopaminergic neurons and, hence, an accumulation of LD and DA metabolites in the brains of PD patients (Bernheimer and Hornykiewicz, 1966; Rajput *et al*., 1997). Quinones, semiquinones, H_2O_2, and other oxyradicals released during the metabolism and auto-oxidation of LD and DA are believed to be toxic to the SNpc neurons. Exogenous LD, which provides symptomatic benefit for PD patients, might also accelerate SN neuronal death.

Several *in vitro* studies have demonstrated the cytotoxicity of LD and DA to a variety of neuronal and non-neuronal cells. DA and LD were first found to be toxic to melanoma

cells, which have a high level of melanin and tyrosinase (Wick, 1979; Wick, 1980). The toxicity of LD and DA has also been found in dopaminergic neurons (Michel and Hefti, 1990), sympathetic neurons (Ziv *et al*., 1994), PC 12 cells (Offen *et al*., 1997; Walkinshaw and Waters, 1995), striatal neurons (Luo, 1998; McLaughlin *et al*., 1998), neuroblastoma cells (Graham, 1978), SN neuroblastoma hybrid cells (Zhang *et al*., 1998), cortical cells (Hoyt *et al*., 1997), and thymocytes (Offen *et al*., 1995). The metabolites of DA and LD may account for this cytotoxicity. The auto-oxidation of these compounds may also be responsible for the observed cytotoxicity.

Despite these *in vitro* studies, very few studies have investigated the ability of LD to cause oxidative stress and oxidative damage *in vivo*. Smith *et al*. (1994) demonstrated that when LD was added to the microdialysate, the •OH content of the effluent from the rat SN increased in a concentration dependent manner, and this was increased further by inhibition of mitochondrial complex I activity. The mechanism of LD induced •OH formation appears to relate to the conversion of LD to DA, since carbidopa prevented LD (0.1mM; 1μM min^{-1})-induced •OH formation in the striatum (Obata and Yamanaka, 1996). When DA is injected into the striatum, cysteinyl-DA complexes were found (Hastings *et al*., 1996) and cell death was prominent (Filloux and Townsend, 1993).

1.3.7.2 Selegiline (SEL)

1.3.7.2.1 Introduction

Monoamine oxidase B (MAO-B) is a predominantly glial enzyme in the brain. The age-related loss of neurons, substituted by glial cells, leads to a significant increase in MAO-B activity. This, in turn significantly contributes to the decline of brain dopaminergic activity with the passing of time (Knoll, 1995). SEL was the first selective inhibitor of MAO-B to be described (Knoll *et al*., 1965; Knoll and Magyar, 1972). It is the internationally used reference substance against which new MAO-B inhibitors are measured. The inhibition of MAO-B by SEL retards the breakdown of DA and enhances

and prolongs the antiparkinsonism effect of levodopa (allowing lower doses of LD to be used). In addition to this, SEL may also help to reduce mild on-off and wearing-off phenomena associated with LD therapy (Aminoff, 2004).

1.3.7.2.2 Pharmacology

1.3.7.2.2.1 Indications

SEL is used as adjunctive therapy for PD patients with a declining or fluctuating response to LD.

1.3.7.2.2.2 Mechanism of action

SEL is an irreversible or "suicide" inhibitor of MAO-B. Irreversible inhibitors initially bind MAO in a reversible, competitive manner, but are then converted by the enzyme to the active inhibitor, which covalently binds the enzyme active site via the FAD cofactor, thus rendering it permanently unavailable for amine metabolism (Foley *et al*., 2000). Its effects can therefore only be overcome by *de novo* synthesis of MAO-B (Abeles and Maycock, 1976).

1.3.7.2.2.3 Adult dosing

Oral treatment with SEL is initially 5 mg in the morning, increasing if necessary to 10 mg in the morning or 5 mg at breakfast and 5 mg at lunch. SEL may cause insomnia when taken later in the day (Gibbon, 2003).

1.3.7.2.2.4 Drug interactions

Within 2-3 days after intiation of SEL therapy, concurrent LD dosage may need to be reduced by 20-50 %. Potentially life-threatening interactions of SEL both with pethidine and with antidepressants (particularly fluoxetine and other SSRIs) have been described. Phenothiazines and butyrophenones, which block striatal DA receptors, should be avoided. Reserpine also interferes with the action of LD and SEL and should be avoided (Gibbons, 2003).

1.3.7.2.2.5 Adverse effects

SEL has a good safety margin and its lack of a "cheese effect" attracted attention even before MAO subtypes had been distinguished (Foley *et al*., 2000). The clinical advantage of the safety of SEL in connection with the treatment of PD is that it can safely be combined with LD therapy. Other MAO inhibitors are contraindicated in LD-treated patients because of the danger of hypertensive crisis (Knoll, 1995). SEL is generally well tolerated. However, hypotension, nausea and vomiting, confusion and agitation may occur (Gibbon, 2003).

1.3.7.2.2.6 Pharmacokinetics

The GIT absorption and tissue distribution of SEL is rapid (Heinonen *et al*., 1989; Waitzinger *et al*., 1996). After the administration of a therapeutic dose of SEL (5-10 mg), peak plasma concentration is reached within 30-120 minutes and 90 % of the dose is bound to plasma proteins (Magyar and Tothfalusi, 1984; Heinonen *et al*., 1989; Mahmood, 1997). It crosses the BBB rapidly (Magyar and Tothfalusi, 1984) and maximal concentrations in the striatum of healthy volunteers are achieved within 5 minutes of intravenous administration (Fowler *et al*., 1987). SEL is metabolized primarily in the liver via the cytochrome P450 system. The major metabolites are N-desmethylselegiline, L-methamphetamine and L-amphetamine (figure 1.16) (Foley *et*

al., 2000). Treatment with SEL is unlikely to result in amphetamine-related effects on neurotransmitter release since the efficacy of L-amphetamine in this respect is only one tenth of that of D-amphetamine (Chiueh and Moore, 1974). The plasma half life of SEL is approximately 30 hours and about 75 % is excreted in the urine and 15 % in the faeces (Gibbon, 2003).

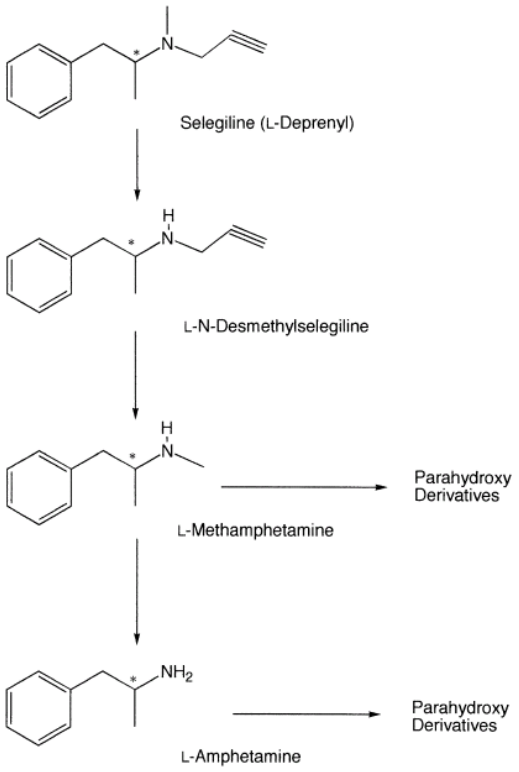

Figure 1.16: The metabolism of SEL to methamphetamine and amphetamine (Foley *et al*., 2000).

1.3.7.2.3 Neuroprotective actions of SEL in PD

SEL, mainly through MAO-B inhibition protects the striatum of monkeys from the neurotoxic effect of MPTP (Cohen *et al*., 1994) but has a neuroprotective effect against intrastriatally injected MPP^+ as well (Wu *et al*., 1995, 1996). SEL also protects the striatum from 6-OHDA-induced toxicity (Knoll, 1978).

Animal studies suggest that chronic SEL treatment results in an increased life expectancy (Milgram *et al*., 1990; Kitani *et al*., 1992; Knoll *et al*., 1995). Birkmayer *et al*. (1975, 1985) indicated that the complementation of LD therapy with SEL increased the life expectancy of PD patients.

The apparent slowing of neurodegenerative processes in PD by SEL may result from a number of different mechanisms but is most often proposed to be due to either an increase in dopaminergic neurotransmission or to neuroprotection against ROS, both of which are mediated by inhibition of MAO-B (Ebadi *et al*., 1996). The MAO-catalyzed oxidation of DA leads to the formation of H_2O_2. Inhibitors of MAO-B would therefore be expected to reduce oxidative stress in dopaminergic neurons by reducing H_2O_2 production. SEL has also been shown to enhance the activity of radical-metabolizing systems in the striatum. For example, the administration of SEL has been shown to result in a significant increase in striatal SOD activity. Two other neuroprotective mechanisms of SEL, independent from MAO-B inhibition, is that the drug has shown to have a neurotrophic factor-like rescuing effect on damaged neurons and also increases reactive astrogliosis (Prochiantz *et al*., 1979). An increase in neuronal survival and an increased reactive astrogliosis may be related since reactive astrocytes are believed to play a role in the survival of damaged neurons, possibly through the provision of astroglially-derived neurotrophic factors.

1.4 RESEARCH OBJECTIVES

The debate about the toxicity of LD to dopaminergic neurons has not yet been resolved. It was discovered through the use of LD by PD patients that, although its initial results were dramatically effective, a growing tolerance to the drug developed. This resulted in a need to increase LD dosages over time. However, treatment with LD, especially at high dosages results in the patients experiencing a number of LD induced motor complications. The metabolic byproducts of LD and DA are believed to be toxic to the nigrostriatal dopaminergic neurons. If this hypothesis is correct, the exogenous administration of LD could alleviate the symptoms of PD in the short term, but accelerate

the disease process in the long term. This in turn could explain the emergence of wearing off effects associated with LD therapy, since this phenomenon is attributed to a reduced interdose presynaptic DA storage capacity (Chase *et al*., 1993; Nutt and Holford, 1996).

Even though the enzymatic and nonenzymatic metabolism of LD and DA can produce ROS and neuronal damage *in vitro*, there has been controversy as to whether these agents can generate oxidative stress and oxidative damage *in vivo.* The main aim of this study is to gain insight into the neuroprotective and neurotoxic roles of DA and LD in the neurodegeneration associated with PD. The study seeks to establish some of the molecular mechanisms by which these catechol compounds exert their neuroprotective or neurotoxic effects, through the employment of various biological and inorganic studies. In addition to this, the study also investigates whether SEL is able to prevent or mitigate the toxic effects of LD and DA and to establish some of the mechanisms by which SEL affords neuroprotection.

The study includes an investigation into the capacity of DA to react with iron, ascorbate and hydrogen peroxide (Fenton reagents) to form 6-OHDA (a potent neurotoxin) *in vitro*. The study also investigates the capacity of DA to generate oxidative stress *in vitro*, characterized by an increase in $O_2^{\bullet -}$ and •OH and a decrease in total GSH. In addition to this, the capacity of DA to cause oxidative damage to lipids (increase in MDA levels) and proteins (increase in carbonyl content) in whole rat brain homogenate will also be investigated. In all these cases, the effects provoked by the presence of Fenton reagents on both the oxidative stress and oxidative damage caused by DA will be investigated. In each study, SEL will be used to determine whether it can quench any free radical effects that may occur.

Furthermore, the study also investigates whether acute LD treatment results in the formation of 6-OHDA in the striatum of male Wistar rats. The striatum is rich in DA, H_2O_2, iron and ascorbate. The generation of •OH via Fenton chemistry is known to convert DA to 6-OHDA *in vitro*. The present study therefore investigates whether exogenous LD administration can accelerate the generation of this neurotoxin in the rat

striatum *in vivo*. In addition to this, the study also investigates the effect of increased striatal iron levels on the ability of LD to generate 6-OHDA in the striatum, and to cause an increase in striatal oxidative stress and oxidative damage. Once again, the co-administration of LD with SEL will be used to investigate whether SEL is able to prevent or reduce any oxidative damage induced by LD. The neuroprotective and neurotoxic effects of LD *in vivo* will be compared to those observed with DA *in vitro*.

It is hoped that the findings of this study will provide additional insight into the nature of the molecular mechanisms involved in the neurotoxicity of LD and DA and contribute towards explaining the progression of PD, associated with an increase in DA turnover in surviving dopaminergic neurons. It is also hoped that the study will contribute to explain the adverse effects associated with LD therapy and provide a foundation for future research on how it may be possible to prevent or mitigate LD's unwanted adverse impacts.

CHAPTER TWO

STABILITY OF 6-HYDROXYDOPAMINE AND ITS FORMATION FROM DOPAMINE

2.1 INTRODUCTION

6-Hydroxydopamine (6-OHDA) is one of the most common neurotoxins used to experimentally model nigral degeneration *in vitro* and *in vivo*. It is selectively taken up into catecholaminergic nerve terminals and destroys them by a mechanism that is generally believed to involve free radical production as well as covalent binding of its semiquinone to macromolecules such as nucleic acids and proteins (Graham *et al*., 1978). Some evidence exists that 6-OHDA is a physiological endogenous neurotoxin. Several studies have reported the presence of 6-OHDA in both rat (Senoh and Witkop, 1959a,b; Senoh *et al*., 1959a,b; Severson *et al*., 1982) and human brain (Curtius *et al*., 1974) as well as in the urine of patients receiving levodopa (LD) treatment (Andrew *et al*., 1993).

The possible pathways of 6-OHDA formation *in vivo* are unclear. Due to the high content of dopamine (DA), hydrogen peroxide (H_2O_2) and free iron in dopaminergic neurons, a non-enzymatic reaction between these elements may possibly lead to endogenous 6-OHDA formation (Slivka and Cohen, 1985; Jellinger *et al*., 1995; Linert *et al*., 1996). Hydroxyl radicals formed by the reaction of H_2O_2 with iron can oxidize the neurotransmitter DA to the neurotoxin 6-OHDA (Jellinger *et al*., 1995). This DA oxidation has been shown to occur at concentrations as low as 50 µM and induces the formation of both 6-OHDA and related quinones (Napolitano *et al*., 1999). Moreover, Liao *et al*. (2003) reported the presence of 6-OHDA in mouse striatum due to the inhibition of monoamine oxidase (MAO) and catechol-0-methyltransferase (COMT) activities. These authors also reported the emergence of a 6-OHDA-like substance in the striatum of methamphetamine-treated rats. 6-OHDA has been detected in rat brain after the administration of 6-hydroxydopa (Karoum *et al*., 1993; Evans and Cohen, 1989),

which differs from LD only in the addition of a hydroxyl group in the 6 carbon position. Evans and Cohen, (1989) and Karoum *et al*. (1993) also reported increased levels of 6-OHDA in the brain following inhibition of MAO with pargyline.

In view of the studies mentioned above, this study sought to investigate the chemical ability of DA to react with iron, ascorbate and H_2O_2 to form 6-OHDA by using a validated high performance liquid chromatography (HPLC) method coupled with an electrochemical detector. In addition to this, due to the high concentration of DA, H_2O_2 and free iron in the striatum and previous studies demonstrating the endogenous formation of 6-OHDA, we decided to investigate whether LD treatment results in the endogenous formation of 6-OHDA in the rat striatum.

In each study selegiline (SEL), a monoamine oxidase B (MAO-B) inhibitor with known antioxidant properties was used to see whether it could reduce yields of 6-OHDA formed. Scavenging of hydroxyl radicals by SEL could suppress yields of 6-OHDA formed. However, SEL blocks the primary metabolic pathway of DA and in addition to this is metabolized to amphetamine and methamphetamine (Meeker and Reynolds, 1990). When taking this into account together with the fact that previous studies have shown an increase in 6-OHDA after MAO inhibition, it suggests that SEL could theoretically result in an increase in 6-OHDA levels. This could cast doubts on its chronic use in Parkinson's disease.

2.2 STABILITY OF 6-HYDROXYDOPAMINE

2.2.1 INTRODUCTION

A potent neurotoxin, 6-OHDA can be produced by hydroxyl radical attack on DA *in vitro* (Slivka and Cohen, 1985; Jellinger *et al*., 1995; Linert *et al*., 1996). It has been hypothesized that the formation of this neurotoxin, *in vivo*, could be implicated in the cause of Parkinson's disease and neurodegeneration produced by some psychostimulants, such as methamphetamine (Seiden and Ricaurte, 1987). Clear evidence for, or against the generation of 6-OHDA *in vivo* after the administration of psychostimulants and other drugs that elevate synaptic concentrations of DA is lacking because the results obtained with these studies are often conflicting (Rollema *et al*., 1986, Marek *et al*., 1990, J. Tong and A.D. Baines, 1993, Liao *et al*., 2003).

Problems with the known rapid auto-oxidation of 6-OHDA at physiological pH (Cohen and Heikkila, 1974; Graham *et al*., 1978) and its stability during sample storage, homogenization and analysis may be responsible for the conflicting results obtained with biological samples. This study therefore examined the stability of 6-OHDA under acidic (0.1 M $HClO_4$), physiological (0.1 M phosphate buffered saline (PBS) at pH 7.4), and alkaline (0.1 M NaOH) pH at room temperature. The study also investigated the stability of 6-OHDA when biological samples and standards were stored at -70 $^{\circ}$C. In addition to this, the study also investigated the effect of added ascorbate on 6-OHDA stability.

2.2.2 MATERIALS AND METHODS

2.2.2.1 Chemicals and reagents

6-Hydroxydopamine hydrobromide was purchased from the Sigma Chemical Corporation, St. Louis, MO, USA. All other chemicals were of the highest quality available and purchased from commercial distributors.

2.2.2.2 Sample preparation

The significance of pH on the stability of 6-OHDA was investigated by examining its stability under basic (0.1 M NaOH), physiological (0.1 M PBS, pH 7.4) and acidic (0.1 M $HClO_4$) pH conditions. Ten micrograms of 6-OHDA was added to 10 ml of each solution (1 µg/ml final concentration) and the solutions were then processed for 6-OHDA at different time periods by injecting 20 µl aliquots into the HPLC system. The effect of ascorbate was evaluated by adding a final concentration of 1 mM of ascorbate to each solution before the addition of the 6-OHDA.

In order to investigate the stability of 6-OHDA during sample homogenization, rats were sacrificed by cervical dislocation and the brains were rapidly excised and homogenized (10 % m/v) in either 0.1 M PBS, pH 7.4 or 0.1 M $HClO_4$. Ten micrograms of 6-OHDA was added to 10 ml of each homogenate. At various time periods, aliquots (0.1 ml) of each homogenate was removed, centrifuged at 12 000 x g for 5 minutes and the supernatant (20 µl) was injected into the HPLC system. Once again the effect of ascorbate was evaluated by adding a final concentration of 1 mM of ascorbate to each homogenate before the addition of the 6-OHDA. The stability of 6-OHDA was also studied in frozen supernatants stored at -70 ºC over a period of two days.

2.2.2.3 Instrumentation

The samples were analyzed on a modular isocratic high performance liquid chromatographic system (Waters Millipore Model 510/Milford; MA, USA). The chromatographic system consisted of a Waters Millipore model 460-electrochemical detector and a Rikadenki model R01 chart recorder (Tokyo, Japan). Samples were introduced into the system using a Rheodyne fixed loop 7725i injector, fitted with a 20 µl loop.

2.2.2.4 Chromatographic conditions

Separation was achieved using an Ultrasphere C18 IP 80A analytical column (5 µm, 250 x 4.6 mm). The mobile phase consisted of an aqueous solution of citric acid (125 mM), sodium phosphate (125 mM), EDTA (100 mg/L), and sodium octylsulfate (30 mg/L). The pH was adjusted to 2.5 with 85 % orthophosphoric acid and was degassed twice using a 0.45 µm membrane filter prior to use. The flow rate was 1 ml/min, and the electrodetection was performed at 0.2 V (Cohen and Slivka, 1985). Only the 6-OHDA was detected at 0.2 V.

2.2.2.5 HPLC method validation

Stock solutions of 6-OHDA were freshly prepared for each test in 0.4 M $HClO_4$ containing 1 mM of ascorbic acid. The analytical procedure was validated by assessment of linearity of calibration (0.1 µg/ml to 1 µg/ml), repeatability, sensitivity, precision and limits of quantification (LOQ) and detection (LOD). To ensure specificity, other hydroxylated dopamine derivatives namely 2-hydroxydopamine and 5-hydroxydopamine were prepared in the same way as the 6-OHDA and injected into the HPLC system. Only the 6-OHDA was detected at 0.2 V. In addition, two concentrations within the calibration range were prepared independently for use as accuracy standards.

2.2.3 RESULTS

2.2.3.1 HPLC method validation

Chromatograms obtained for 6-OHDA show symmetrical, well resolved peaks with a retention time of 9.6 minutes. Figure 2.1 shows a typical chromatogram of 6-OHDA.

Figure 2.1: A typical chromatogram obtained for 6-OHDA (1 µg/ml)

Regression analysis shows that the concentration and peak height ratio are linear over the concentration range studied, with an excellent correlation coefficient of $r^2 = 0.9998$. Each point on the calibration curve is based on triplicate determinations repeated five times. Figure 2.2 represents a typical calibration curve obtained after plotting the mean currents of the peaks versus concentration.

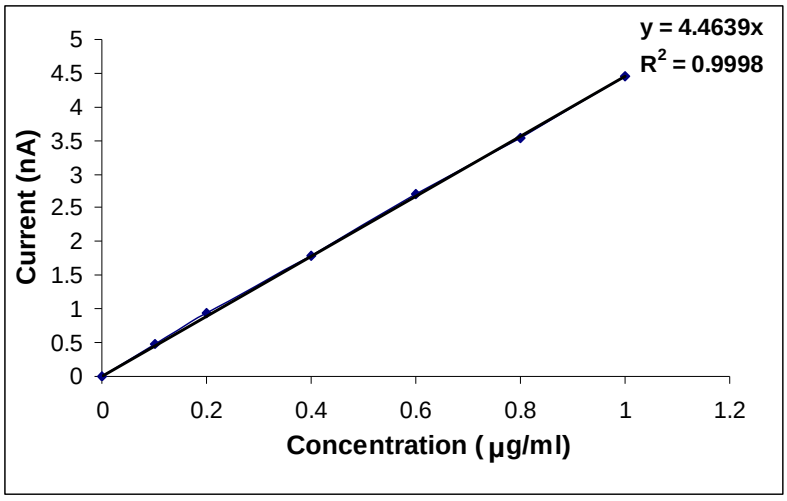

Figure 2.2: A typical calibration curve obtained for 6-OHDA.

Sensitivity of the method was evaluated by determining the lowest reproducible concentration of 6-OHDA detectable. The LOQ and LOD were determined as 0.0125 µg/ml and 0.00625 µg/ml respectively. The precision is reported as percentage relative standard deviation (% RSD). The interday precision ranges from 0.19 % to 2.2 % (table 2.1) while the intraday precision ranges from 0.86 % to 3.55 % (table 2.1) for the concentration range studied.

Table 2.1: 6-OHDA validation data

Concentration	Relative Standard Deviation (%)	
(µg/ml)	Interday (n=5)	Intraday (n=5)
0.1	0.19	0.86
0.2	0.74	0.86
0.4	0.81	1.81
0.6	1.98	1.59
0.8	2.20	0.63
1	2.20	3.55

2.2.3.2 Stability of 6-OHDA

Under alkaline conditions (0.1 M NaOH), 6-OHDA was extremely unstable and no peaks were detectable electrochemically. Figure 2.3 is a series of chromatograms showing how the 9.6 minute peak of 6-OHDA decreased over a 48 hour period when a freshly prepared solution of 6-OHDA in 0.1 M $HClO_4$ was used.

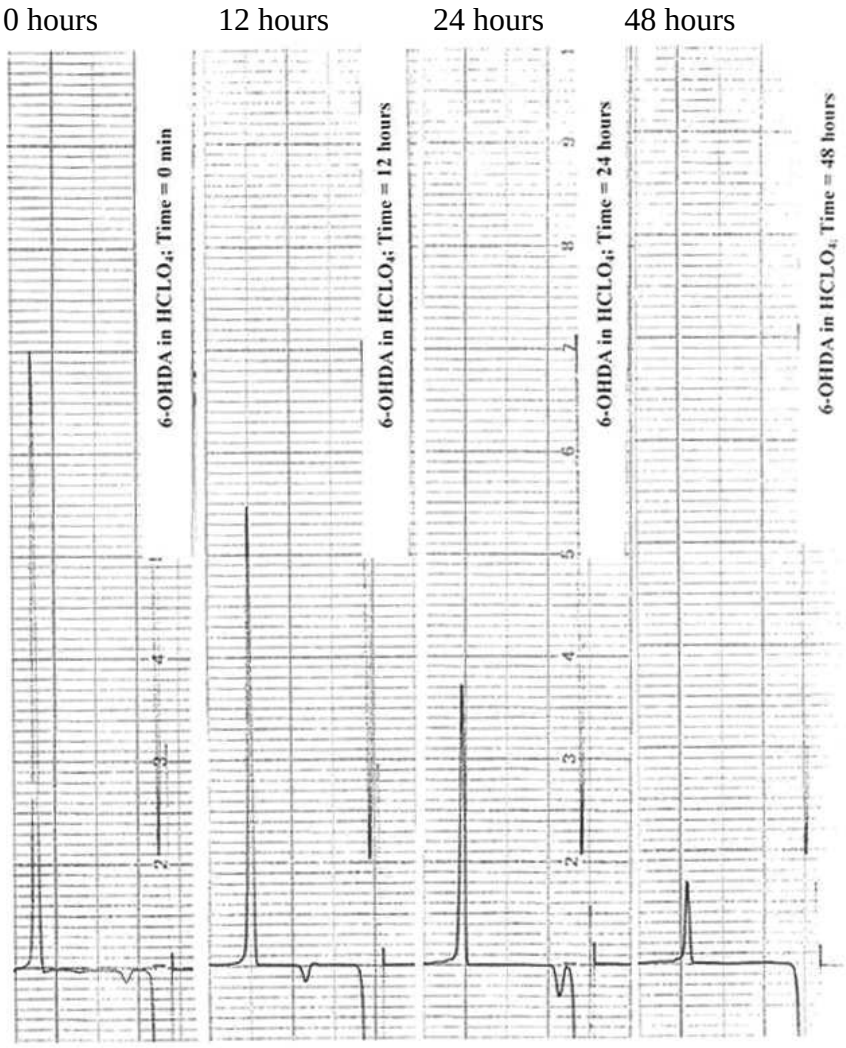

Figure 2.3: A series of chromatograms showing the disappearance of 6-OHDA over a 48 hour period when 6-OHDA was added to a solution of 0.1 M $HClO_4$.

The percentage of 6-OHDA remaining at different times is shown in figure 2.4. The graph shows that only about 20 % of the 6-OHDA remained after 48 hours.

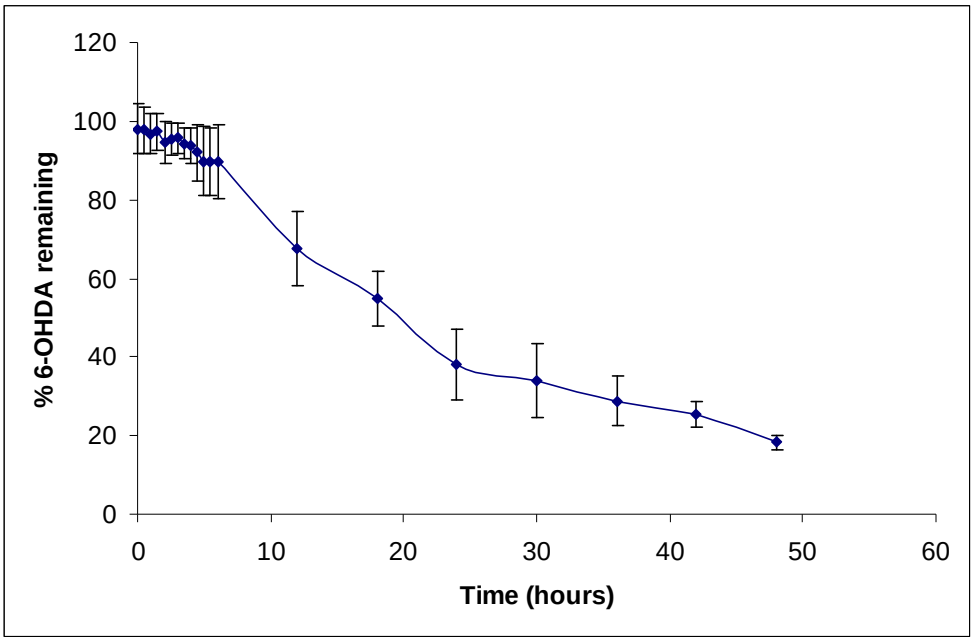

Figure 2.4: Graph showing the percentage of 6-OHDA remaining at different time periods when 6-OHDA was added to a solution of 0.1 M $HClO_4$.

At physiological pH (0.1 M PBS, pH 7.4) the 9.6 minute peak of 6-OHDA decreased more rapidly and no peaks were detected electrochemically after 2 hours. Figure 2.5 is a series of chromatograms showing the disappearance of the 9.6 minute peak over a 1.5 hour period.

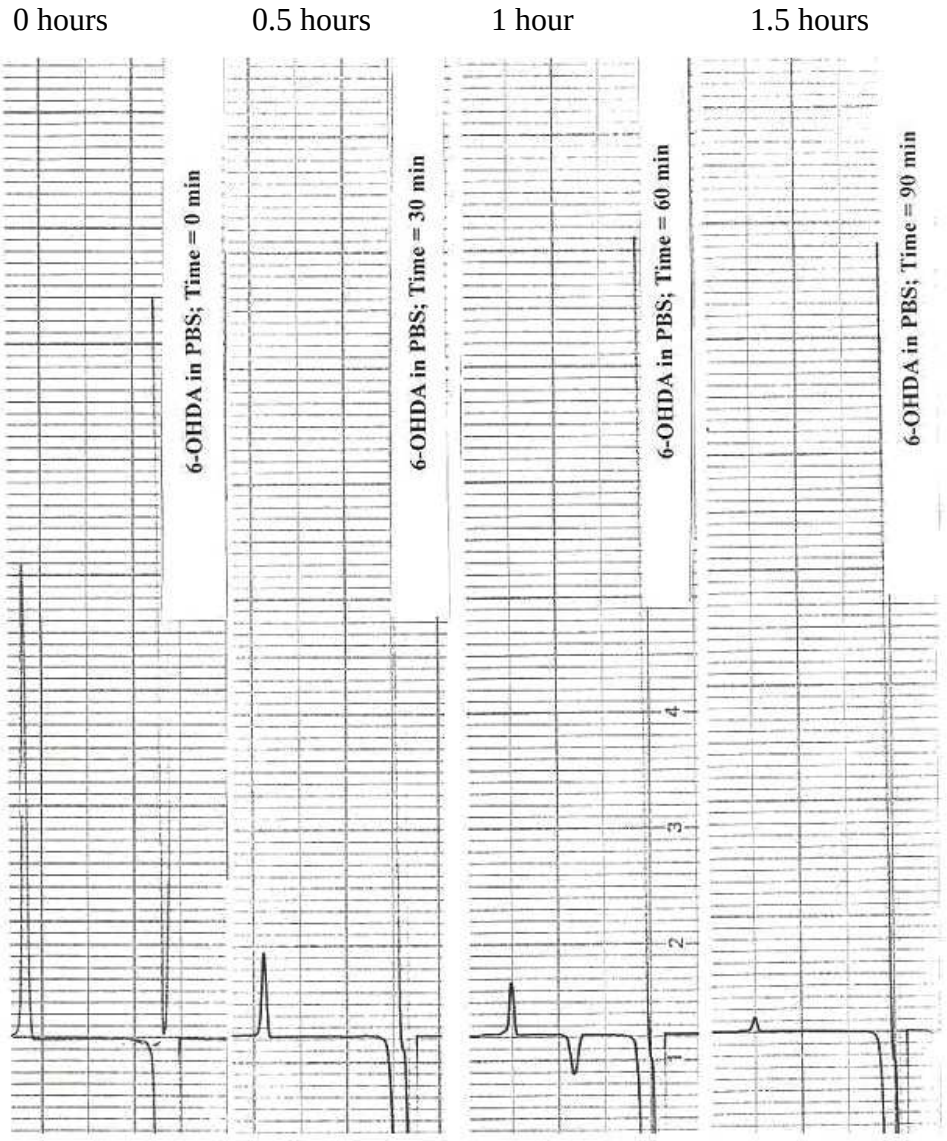

Figure 2.5: A series of chromatograms showing the disappearance of 6-OHDA over a 1.5 hour period when 6-OHDA was added to a 0.1 M solution of PBS, pH 7.4.

Figure 2.6 shows that 6-OHDA is very unstable at physiological pH and that only about 2 % of the 6-OHDA remained after 2 hours.

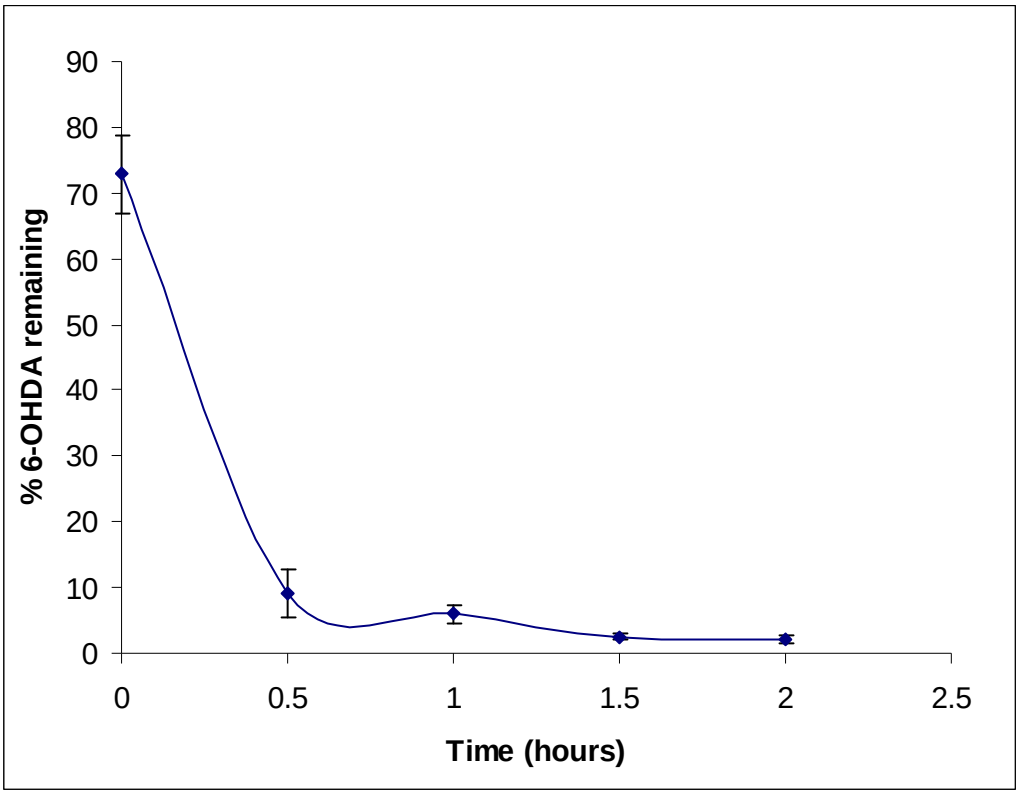

Figure 2.6: Graph showing the percentage of 6-OHDA remaining at different time periods when 6-OHDA was added to a solution of 0.1 M PBS, pH 7.4.

The addition of 1 mmol/L of ascorbic acid to a solution of 6-OHDA in 0.1 M $HClO_4$ slowed the disappearance of the 6-OHDA peak so that 60 % remained after 48 hours (figure 2.7). The addition of 1 mmol/L of ascorbic acid to the PBS also prolonged the life of the 6-OHDA peak so that 18 % of 6-OHDA remained after 6 hours and 3 % of the 6-OHDA remained after 48 hours (figure 2.8).

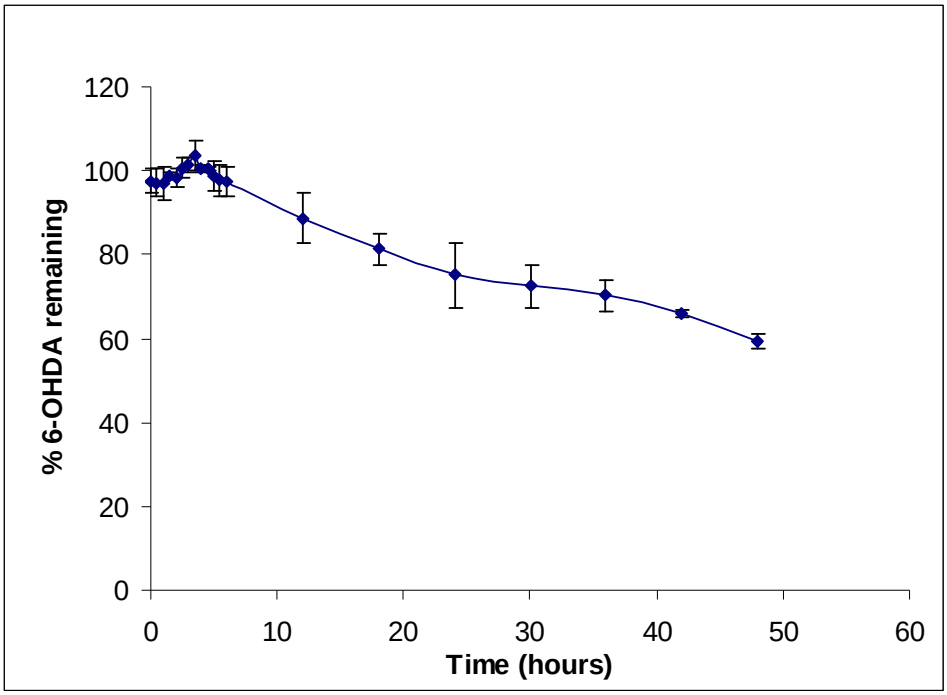

Figure 2.7: Graph showing the percentage of 6-OHDA remaining at different time periods when ascorbate (1 mM) was added to a solution of 6-OHDA in 0.1 M $HClO_4$.

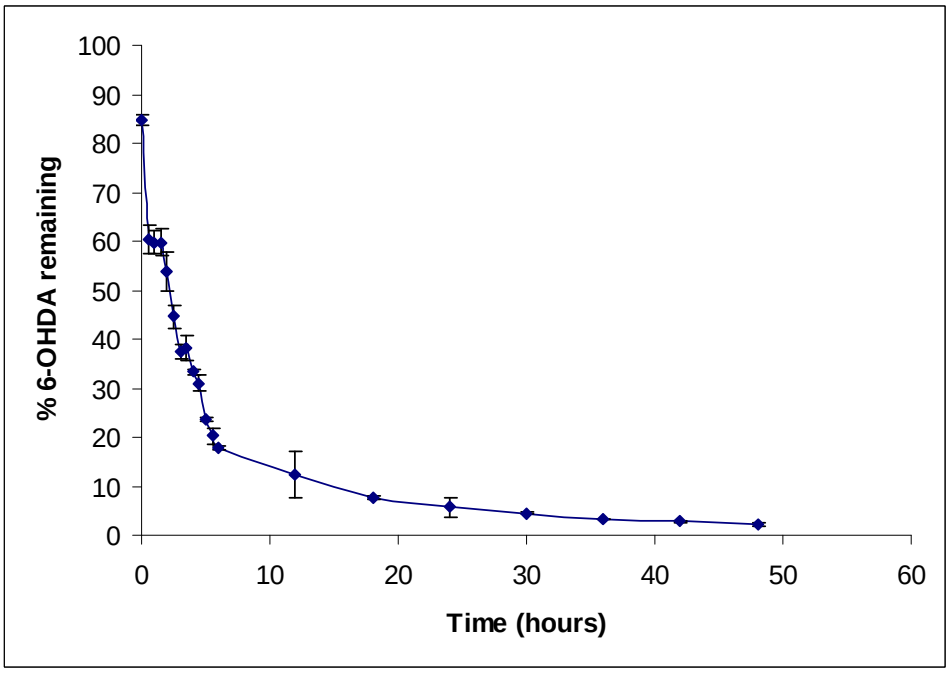

Figure 2.8: Graph showing the percentage of 6-OHDA remaining at different time periods when ascorbate (1 mM) was added to a solution of 6-OHDA in 0.1 M PBS.

As shown in figure 2.9, 6-OHDA was less stable in the samples containing rat brain tissue homogenized in $HClO_4$ than samples containing no tissue homogenate. In the samples containing tissue homogenate no peak for 6-OHDA was detected after 36 hours. However, the addition of 1 mM of ascorbate to the tissue homogenate increased the stability of 6-OHDA so that the amounts of 6-OHDA measured in the samples containing both tissue homogenate and exogenous 6-OHDA were not significantly different from those obtained in the samples containing no tissue extract.

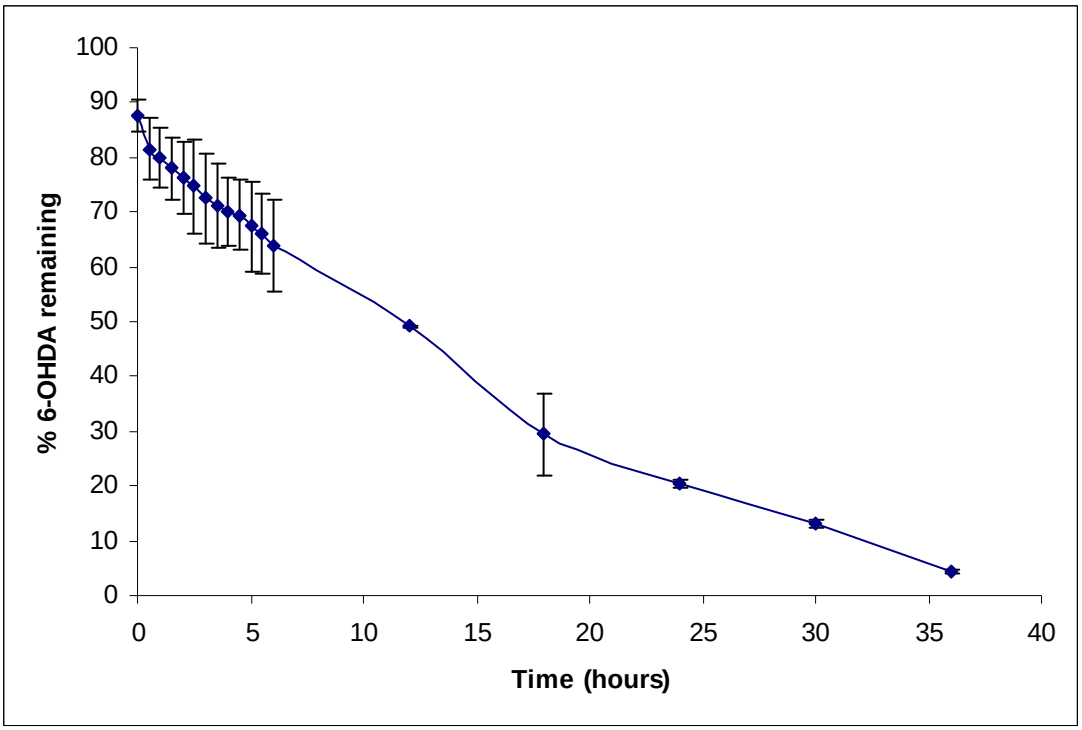

Figure 2.9: Graph showing the percentage of 6-OHDA remaining at different time periods when 6-OHDA was added to rat brain homogenized in 0.1 M $HClO_4$.

The amounts of 6-OHDA measured in samples containing both rat brain tissue homogenized in PBS and exogenous 6-OHDA are significantly higher than those obtained in the samples containing no homogenate. Figure 2.10 shows that approximately 5 % of the 6-OHDA remained after 30 hours.

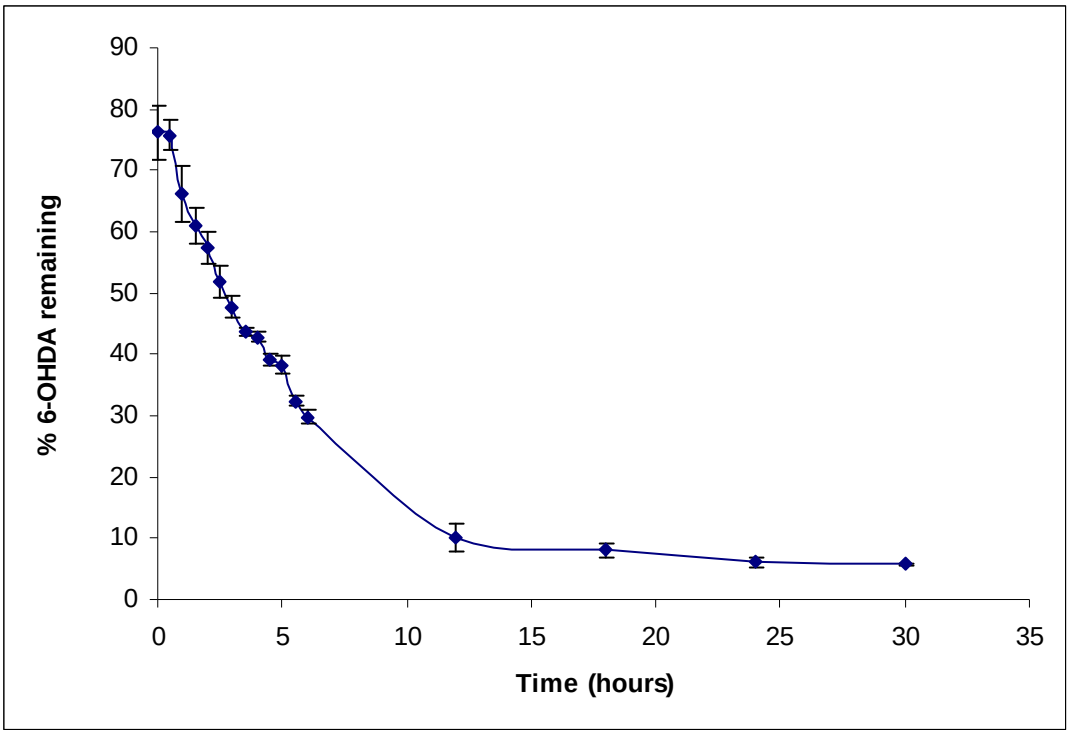

Figure 2.10: Graph showing the percentage of 6-OHDA remaining at different time periods when 6-OHDA was added to rat brain homogenized in 0.1 M PBS.

No change in peak heights was observed in samples prepared from brain supernatants (PBS and $HClO_4$) stored at -70 $^{\circ}$C for a period of 2 days.

2.2.4 DISCUSSION

This study confirms the previously published observation that 6-OHDA undergoes rapid auto-oxidation at physiological pH (0.1 M PBS, pH 7.4) *in vitro*. The 6-OHDA generated in the rat brain may therefore also be very short lived and difficult to detect. Increasing the ascorbate concentration in the PBS to 1 mM increased the stability of 6-OHDA. Relatively high concentrations of ascorbate are therefore needed to maintain 6-OHDA in the reduced state. Ascorbate may increase the stability of 6-OHDA by promoting the redox cycling of the quinone oxidation products of 6-OHDA back to the parent compound. The stability of 6-OHDA generated *in vivo* may therefore be affected by local tissue levels of ascorbate and other anti-oxidant molecules such as tocopherol. Ascorbic

acid is present in relatively large concentration in neural tissue; with mean levels in the range of 1-2 mM in rat brain (Milby *et al*., 1982). Higher mean levels of 8.8 ± 1.1 mM are reported for nerve terminals from rat brain (Kuo *et al*., 1978). The presence of endogenous ascorbate and other anti-oxidant molecules in the rat brain homogenate may account for the increased stability of 6-OHDA observed in samples that contained both rat brain tissue homogenate and exogenous 6-OHDA when compared to samples that contained no tissue homogenate. A number of other factors can also affect the observed stability of 6-OHDA *in vivo*, such as storage in synaptic vesicles and the continued synthesis of 6-OHDA from endogenous DA.

The results of this study also show that the stability of 6-OHDA under acidic pH (0.1 M $HClO_4$) is much greater than that observed under physiological pH. 6-OHDA is more stable in samples that contained no tissue homogenate than samples that contained tissue homogenized in $HClO_4$. This was therefore opposite to what was observed under physiological pH. A possible explanation for this reduced stability is that oxidized 6-OHDA is highly reactive with nucleophiles. Oxidized 6-OHDA could therefore have bound covalently to nucleophilic groups in albumin and other proteins (Saner and Thoenen, 1971) present in the homogenate. When the ascorbate concentration was increased to 1 mM in the tissue homogenate, the stability of 6-OHDA in samples containing ascorbate (1 mM), tissue homogenate and exogenous 6-OHDA was not significantly different from the stability of 6-OHDA observed in samples containing no tissue homogenate.

In subsequent experiments striatal samples that were to be processed for 6-OHDA were homogenized in 0.4 M $HClO_4$ containing 1 mM of ascorbate in order to maintain any 6-OHDA formed in the reduced state as long as possible.

Storage of brain supernatants at -70 ºC for 2 days had no observable effect on the peak height of 6-OHDA. In future experiments striatal samples to be processed for 6-OHDA were rapidly excised, frozen in liquid nitrogen and stored at -70 ºC for a period not exceeding 2 days.

It is clear that the detection of any 6-OHDA formed after LD treatment depends on a large number of factors, including local tissue levels of ascorbate, iron, DA, as well as levels of glutathione and tocopherol and other anti-oxidant enzymes. However, the main aim of this study was to help prevent the loss of 6-OHDA during sample storage, homogenization and analysis in future experiments.

2.3 EFFECT OF SELEGILINE ON THE *IN VITRO* FORMATION OF 6-HYDROXYDOPAMINE

2.3.1 INTRODUCTION

Hydroxyl radicals (•OH) are highly oxidizing species that have been implicated as mediators of tissue damage in a number of *in vitro* and *in vivo* studies. A classical source of •OH is from the univalent reduction of H_2O_2 by metal ions, first reported by Fenton (Slivka and Cohen, 1985). The Fenton reaction between a ferrous salt or ferrous chelate and H_2O_2 can be represented by the overall equation:

$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^- + \bullet OH$ (Equation 2.1)

In cellular systems, the ferric ions (Fe^{3+}) that are generated in the Fenton reaction (equation 2.1) are recycled by suitable reducing agents, for example ascorbic acid (equation 2.2). Ferric ions also oxidize catechols or superoxide anions, resulting in the formation of reduced ferrous ions (equation 2.3). The recycling of iron between its ferric and ferrous states permits the generation of •OH to continue until the source of reducing equivalents (or H_2O_2) is expended.

$2Fe^{3+} + AH_2 \rightarrow 2Fe^{2+} + A$ (Equation 2.2)

$Fe^{3+} + O_2^{\bullet -} \rightarrow Fe^{2+} + O_2$ (Equation 2.3)

Evidence for the existence of •OH in biochemical systems is based on the identification of products formed when •OH reacts with an exogenous compound that has been added to the system in relatively high concentrations. Previous studies have shown that •OH will engage in the ring hydroxylation of a variety of aromatic compounds including phenol and substituted phenols (Richmond *et al*., 1981). For example, •OH reacts with salicylate to generate 2,3 and 2,5-dihydroxybenzoic acid which can be measured electrochemically using high performance liquid chromatography (Obata and

Yamamaka,1996). DA is an endogenous catecholamine that is found in high concentration in the striatum. Senoh *et al*. (1959) reported that in the presence of Fe(II)-EDTA and ascorbate, DA could be ring-hydroxylated to form a product that appeared to be 6-OHDA. The rate constant for the reaction of •OH with DA is 5.9 X 10^9 $M^{-1}s^{-1}$ at a pH of 4.7 (Slivka and Cohen, 1985).

In the present study, hydroxyl radicals were generated in the presence of DA at a pH of 7.4 by using the Fe(II)-EDTA/H_2O_2 and ascorbate system (Fenton system) as the •OH generating system and a validated high performance liquid chromatography with electrochemical detection (HPLC-ECD) method was used to investigate whether 6-OHDA was formed. In addition, the study also investigated whether SEL was able to suppress yields of 6-OHDA formed.

2.3.2 MATERIALS AND METHODS

2.3.2.1 Chemicals and reagents

DA hydrochloride, 6-OHDA hydrobromide, SEL hydrochloride, sodium octylsulfate, sodium phosphate and citric acid were purchased from the Sigma Chemical Corporation, St. Louis, MO, USA. EDTA and ferrous (II) sulfate were purchased from Merck, Darmstadt, Germany. All other bench reagents were purchased from Saarchem(Pty). Ltd, Krugersdorp, South Africa.

2.3.2.2 Hydroxyl radical generating system

To determine the hydroxylation of DA, Fe(II)-EDTA/H_2O_2 and ascorbate (Fenton system) was used as the hydroxyl radical generating system. This reaction system is based on a coupled reaction in which ferrous chelates react with H_2O_2 to give rise to hydroxyl radicals according to the following equations.

$FeCl_2 + EDTA \rightarrow Fe^{2+}$ -EDTA (Equation 2.4)

Fe^{2+} -EDTA + $H_2O_2 \rightarrow Fe^{3+}$ -EDTA + •OH + OH^- (Equation 2.5)

Fe^{3+} -EDTA + ascorbic acid $\rightarrow Fe^{2+}$ -EDTA + oxidized ascorbic acid (Equation 2.6)

The first reaction (equation 2.4) results in the formation of a ferrous chelate. The ferrous chelate then produces hydroxyl radicals by reacting with H_2O_2 in the second reaction (equation 2.5). The chelated Fe(III)–EDTA is then reduced to the ferrous state by the third reaction (equation 2.6). In this process, the ferrous irons are constantly regenerated in a coupled reaction in which the ferric ions are reduced by ascorbic acid. This results in a reliable and continuous production of hydroxyl radicals throughout the experiment. The sequence of additions and final concentrations of reagents for the Fenton system were as follows: water, phosphate buffer (final concentration 50 mM, pH 7.2), H_2O_2 (100 µM), DA (1 mM), ascorbate (1 mM), EDTA (240 µM) and ferrous sulfate (200 µM). The effect of SEL was evaluated by adding various concentrations (0.1, 1 and 10 mM) before the addition of DA. The reaction mixture was incubated at room temperature with intermittent stirring. At various time periods (10, 30, 60, 120 minutes), aliquots (0.1 ml) of reaction mixture were removed and quenched in ice cold 0.4 M $HClO_4$ containing 1 mM ascorbate. The ascorbate was necessary to maintain the 6-OHDA in the reduced state. The reaction time was 120 minutes. The samples were analysed for 6-OHDA using high performance liquid chromatography with electrochemical detection. Final results are expressed as µg of 6-OHDA/10 ml.

2.3.2.3 Instrumentation

As described in section 2.2.2.3

2.3.2.4 Chromatographic conditions

As described in section 2.2.2.4

2.3.2.5 Statistical analysis

The differences in the means were analysed using a one way analysis of variance (ANOVA) followed by the Student Newman-Keuls Multiple Range Test. The level of significance was set at $p < 0.05$.

2.3.3 RESULTS

Figure 2.11 is a series of chromatograms showing that when the reaction mixture was quenched by dilution into 0.4 M $HClO_4$ containing 1 mM ascorbate, and analysed at +0.2 V, a peak with chromatographic and electrochemical properties identical to 6-OHDA was formed.

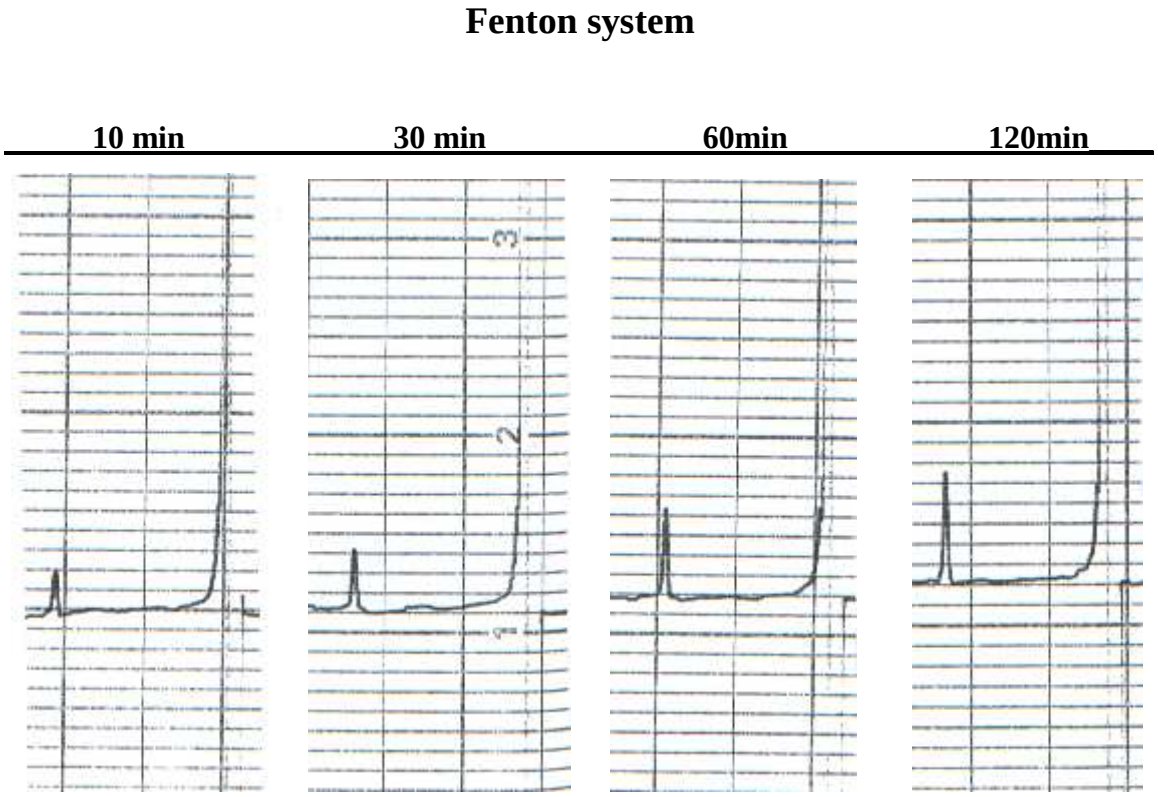

Figure 2.11: A series of chromatograms showing 6-OHDA formation over time when DA is incubated with the Fenton system (0.1 ml aliquots of the reaction mixture were quenched in 0.9 ml of 0.4 M $HClO_4$.)

The yield of 6-OHDA formed after a reaction time of 120 minutes using the Fenton system is shown in table 2.2. As evident in table 2.2 and figure 2.12 SEL suppressed the yield of 6-OHDA formed in a concentration dependant manner with the 10 mM SEL concentration reducing the yield of 6-OHDA to 15.4 % when compared to the control.

Table 2.2: Suppression of the yield of 6-OHDA formed in the Fenton system by SEL. DA was present at 1 mM. The reaction time was 120 minutes. Values are mean ± SD for n=3. Values in parenthesis are percent control. $^{*}(p < 0.001)$ versus control (ANOVA followed by Student-Newman-Keuls Multiple range test).

Scavenger (mM)	6-OHDA (µg/10 ml)
None (control)	12.11 ± 0.39 (100 %)
SEL	
0.1	9.34 ± 0.13 (77.1 %)*
1.0	3.89 ± 0.79 (32.1 %)*
10	1.89 ± 0.26 (15.6 %)*

Figure 2.12 shows 6-OHDA formation with time in the Fenton system. Product formation was continuous in the Fenton system, but was greatly reduced when SEL was added to the reaction mixture.

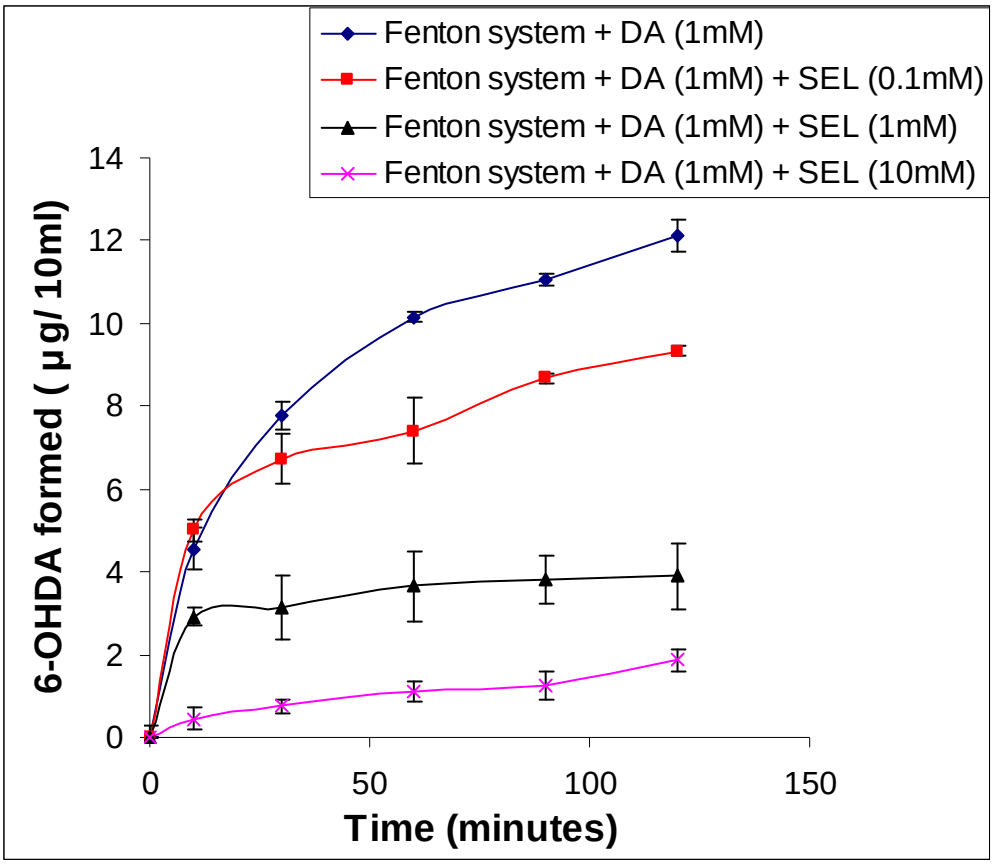

Figure 2.12: Showing the effect of SEL on 6-OHDA formation with time in the Fenton system.

2.3.4 DISCUSSION

Important observations made during this study include (i) the chemical ability of DA to react with iron, ascorbate and H_2O_2 to form 6-OHDA in a cell free environment, indicating a non-enzymatic reaction and (ii) the ability of SEL to suppress yields of 6-OHDA formed.

The fact that 6-OHDA can be formed in the presence of iron, H_2O_2 and ascorbate *in vitro* suggests that 6-OHDA may be formed from DA in the central nervous system *in vivo*. Striatal dopaminergic neurons contain significant levels of DA, H_2O_2 and free iron. This may create a chemical environment which would allow for the conversion of DA to

6-OHDA. The formation of this neurotoxin may then result in cellular damage to DA neurons (Slivka and Cohen, 1985; Jellinger *et al*., 1995; Linert *et al*., 1996).

The hydroxylation of various aromatic compounds has been used as a indicator of •OH generation by a number of different investigators (Halliwell, 1978; Raghavan and Steenken, 1980; Sloot and Gramsbergen, 1995). The ring hydroxylation of DA by •OH results in the formation of 6-OHDA. The ability of SEL to inhibit the formation of 6-OHDA may be due to its known hydroxyl radical scavenging properties (Tan *et al*., 1993; Thomas *et al*., 1997). The ability of SEL to bind to iron will be investigated in a later chapter.

One of the remarkable pharmacological effects of SEL is that it protects the striatum from 6-OHDA induced toxicity. The inhibition of the uptake of 6-OHDA into nigrostriatal dopaminergic neurons plays an important role in the neuroprotective role of SEL against the toxicity of this specific neurotoxin. This study also shows that in addition to this, SEL may also protect against 6-OHDA toxicity by inhibiting the formation of this toxin. On the other hand, the fact that SEL inhibits 6-OHDA formation *in vitro* does not necessarily mean that this will be the case *in vivo.* For example, SEL is a selective MAO-B inhibitor and the inhibition of this enzyme may actually favour the formation of 6-OHDA (Liao *et al*., 2003). SEL is also metabolized to amphetamine and methamphetamine (Meeker and Reynolds, 1990). Liao *et al*. (2003) reported that high doses of methamphetamine (50 mg/kg) results in 6-OHDA formation in the striatum of mice.

The effect of SEL on 6-OHDA formation *in vivo* was investigated in the following experiment.

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2.4 EFFECT OF A SINGLE LARGE DOSE OF L-DOPA AND SELEGILINE ON STRIATAL 6-HYDROXYDOPAMINE FORMATION *IN VIVO*

2.4.1 INTRODUCTION

Parkinson's disease (PD), first described by James Parkinson in 1817 (Parkinson, 1817) is characterized by a marked loss of pigmented dopaminergic neurons from the *substantia nigra pars compacta* (SNpc), a midbrain structure. In 1960, Hornykiewicz and coworkers discovered a significant loss of DA in the striatum of PD patients (Ehringer and Hornykiewicz, 1960). Since DA itself could not cross the blood brain barrier, Hornykiewicz and colleagues predicted that levodopa (LD), the precursor of DA would improve parkinsonian symptoms. One year later, Birkmayer and Hornykiewicz, (1961) reported marked therapeutic success in the treatment of PD after intravenous administration of LD. By 1967, Cotzias and coworkers (Cotzias *et al*., 1967) were able to announce that large oral doses of LD resulted in marked improvement of PD. Although LD was initially very effective in treating the symptoms of the disease, a growing tolerance to the drug developed and it failed to halt the underlying progression of the disease. Eventually, side effects such as dyskinesias (abnormal movements), gastrointestinal symptoms, insomnia, hallucinations and psychosis became worse than the benefits of the drug (Lees, 1995; Katzenschlager and Lees, 2002).

A possible mechanism by which LD may accelerate the disease process and result in a variety of side effects is that exogenous LD administration may significantly increase the formation of 6-OHDA. There is a substantial amount of evidence implicating the involvement of free radicals as one of the major causes of nigral degeneration in Parkinson's disease. The previous experiment demonstrated that free hydroxyl radicals can react with DA to form 6-OHDA. The main aim of this study was to use a validated HPLC-ECD method to search for the presence and to assess the formation of 6-OHDA after the administration of a single large dose of LD (together with a dopa decarboxylase inhibitor, benserazide) in the absence and presence of the MAO-B inhibitor, SEL.

2.4.2 MATERIALS AND METHODS

2.4.2.1 Chemicals and reagents

L-3,4-dihydroxyphenylalanine (LD), benserazide hydrochloride, SEL hydrochloride, sodium octylsulfate, sodium phosphate and citric acid were purchased from the Sigma Chemical Corporation, St. Louis, MO, USA. EDTA and ferrous (II) sulfate were purchased from Merck, Darmstadt, Germany. All other bench reagents were purchased from Saarchem(Pty). Ltd, Krugersdorp, South Africa. LD was dissolved in saline containing 0.2 mg/ml of ascorbic acid and 0.1 M HCl, the solution was mixed until clear and the pH was adjusted to 6.2 with 0.1 M disodium hydrogen phosphate. SEL and benserazide hydrochloride were made up in physiological saline.

2.4.2.2 Animals

Adult male Wistar rats were used in this study and were housed and maintained as described in appendix one

2.4.2.3 Drug treatment

Rats were divided into four groups (I – IV), with fifteen rats in each group. Rats were then treated as described in table 2.3. Group I served as the control and received saline, Group II received LD (50 mg/kg) and Group III received 10 mg/kg of SEL. Group IV received a combination of LD (50 mg/kg) and SEL (10 mg/kg). Each animal received a dose volume of 1 µl/g of body weight of LD and/or SEL i.p. as a single large dose. LD treatment groups also received 12.5 mg/kg of benserazide prepared in saline to prevent the peripheral decarboxylation of LD. Five rats from each group were sacrificed by decapitation at 60, 120 and 180 minutes after initial treatment. The striatum of each rat

was removed, frozen in liquid nitrogen and stored at -70 °C, for a period not exceeding 2 days.

Table 2.3: Treatment schedules used to test the ability of LD and SEL to induce the endogenous formation of 6-OHDA in the rat striatum.

Treatment group	Dose (i.p.)	Time (min) of decapitation after initial treatment
Control (n = 15)	Saline	60 (n = 5)
		120 (n = 5)
		180 (n = 5)
LD (n = 15)	50mg/kg	60 (n = 5)
		120 (n = 5)
		180 (n = 5)
SEL (n = 15)	10mg/kg	60 (n = 5)
		120 (n = 5)
		180 (n = 5)
LD	50mg/kg	60 (n = 5)
and SEL (n=15)	10mg/kg	120 (n = 5)
		180 (n = 5)

2.4.2.4 Preparation of tissue for HPLC analysis

On the day of the experiment, the striatal samples were thawed on ice, weighed and sonicated (50 Hz for 60 s) in ice cold 0.4 M $HClO_4$ containing 1 mM of ascorbate using an ultrasonic cell disruptor (1 mg of tissue in 10 µl). The sonicated samples were kept on ice for 30 minutes and centrifuged at 10 000 x g for 10 minutes using a bench top centrifuge. The supernatant (20 µl) was injected directly into the HPLC-ECD system for analysis.

2.4.2.5 Instrumentation

As described in section 2.2.2.3

2.4.2.6 Chromatographic conditions

As described in section 2.2.2.4

2.4.2.7 Statistical analysis

As described in section 2.3.2.5

2.4.3 RESULTS

Experimental designs and treatment schedules used for the search for 6-OHDA after the administration of a single large dose of LD and/or SEL are summarized in table 2.3. No detectable amount of 6-OHDA was found in the striatum after any of these treatments.

2.4.4 DISCUSSION

The axon terminals of dopaminergic neurons in the striatum are highly enriched in the neurotransmitter, DA, with concentrations estimated at 8 mg/g (or 47 mM) (Anden *et al*., 1966). H_2O_2 is formed within these neurons during the catabolism of DA by mitochondrial MAO. The rate of H_2O_2 production should follow neuronal activity and the rate of turnover of DA. In addition to this, ascorbic acid is also present at high concentrations in the complex environment of brain tissue. Mean levels are in the range of 1-2 mM in rat brain (Milby *et al*., 1982) and 0.4-1.3 mM in major divisions of the human brain (Mefford *et al*., 1981). Higher mean levels of 8.8 ± 1.1 mM are reported for nerve terminals in rat brain (Kuo *et al*., 1979). Iron is also found in high concentrations in several regions of the basal ganglia including the *substantia nigra*, the *globus pallidus* and the *putamen* (Dexter *et al*, 1989a,b). As discussed in the previous experiment, the endogenous presence of the above reactants in the rat brain could have created a chemical environment that allowed DA to be converted to 6-OHDA.

The previous experiment confirmed the observation by Slivka and Cohen, (1985) that hydroxyl radicals can convert DA to 6-OHDA *in vitro*. However, the products of this reaction were not detected *in vivo*. Our negative finding suggests, therefore, that 6-OHDA is not produced *in vivo* under the present conditions. Although 6-OHDA is known to auto-oxidize to stable quinone products *in vitro*, the results of the stability study conducted in section 2.2 suggest that the preparation procedures used during this experiment did not result in a significant loss of 6-OHDA.

A possible reason for the failure to detect 6-OHDA in the striatum may be that the 6-OHDA formed in the striatum is very short lived. 6-OHDA undergoes rapid auto-oxidation to quinone products at physiological pH. The quinone oxidation products of 6-OHDA are highly reactive with nucleophiles, such as sulfhydryl groups in glutathione (GSH) and other proteins (Tong and Baines, 1993). Nucleophilic attack by sulfhydryl groups in GSH and other proteins could scavenge the oxidized products of 6-OHDA as rapidly as they were produced (Tong and Baines, 1993). This in turn would prevent the redox cycling of these quinone products back to 6-OHDA by endogenous ascorbate. Rapid scavenging of oxidized 6-OHDA may therefore account for the absence of detectable levels of 6-OHDA in the striatal samples of rats. Another explanation is that LD and SEL treatment may produce concentrations of 6-OHDA that are below the limit of detection of the assay.

The duration of treatment, i.e. giving a single dose of LD and/or SEL could also have affected the outcome of the investigation. The following experiment investigates whether treatment with LD and/or SEL over a longer period of time results in 6-OHDA formation. 6-OHDA is also more likely to occur under pro-oxidant conditions. The following experiment therefore also investigates the effect of elevated iron levels in the striatum on 6-OHDA formation. PD is associated with increased iron levels and reduced antioxidant defense levels in the striatum.

2.5 EFFECT OF ACUTE L-DOPA AND SELEGILINE TREATMENT IN IRON INFUSED RATS ON STRIATAL 6-HYDROXYDOPAMINE FORMATION *IN VIVO*

2.5.1 INTRODUCTION

The previous experiment demonstrated that 6-OHDA is not produced *in vivo* after the administration of a single large dose of LD and/or SEL. The inability to detect 6-OHDA after the administration of a single dose of these drugs is consistent with that of a previous study using HPLC-ECD analysis (Karoum *et al*., 1993)

The main objective of this study is to investigate whether increasing the duration of treatment with LD and/or SEL would favour striatal 6-OHDA formation. In addition to this, the study also focuses on the effect of increased free iron levels on 6-OHDA formation. Iron levels in the striatum are not only higher than in other regions of the brain (Gerlach *et al*., 1994), but are increased approximately 35 % in PD patients compared to age-matched controls (Dexter., 1989a). Rats were therefore given an intrastriatal infusion of ferrous sulfate prior to treatment with LD and/or SEL. An increase in free iron would result in increased free radical production via the Fenton reaction, thus amplifying the chemical reaction between hydroxyl radicals and DA resulting in 6-OHDA formation.

2.5.2 MATERIALS AND METHODS

2.5.2.1 Chemicals and reagents

As described in section 2.4.2.1

2.5.2.2 Animals

Adult male Wistar rats were used in this study and were housed and maintained as described in appendix one.

2.5.2.3 Drug treatment

Animals were divided into two treatment regimes. Animals in treatment regime 2 received an intrastriatal infusion of iron (II) sulfate before the i.p. administration of LD and/or SEL, while rats in treatment regime 1 only received LD and/or SEL treatment without an intrastriatal infusion of iron (II) sulfate.

2.5.2.3.1 Treatment regime 1

Rats were divided into four groups (I – IV), with five rats in each group. Group I served as the control and received saline, Group II received LD, 10 mg/kg/bd and Group III received 2.5 mg/kg/bd of SEL. Group IV received a combination of LD (10 mg/kg/bd) and SEL (2.5 mg/kg/bd). Each animal received a dose volume of 1 µl/g of body weight of LD and/or SEL i.p. twice daily for 7 days. LD treatment groups also received 2.5 mg/kg/bd of benserazide prepared in saline to prevent the peripheral decarboxylation of LD. The animals were sacrificed by cervical dislocation on the 8^{th} day. The striatum of each rat was removed, frozen in liquid nitrogen and stored at -70 $^{\circ}$C.

2.5.2.3.2 Treatment regime 2

Male Wistar rats were anaesthetized using diethyl ether. Two microliters (2 µl) of ferrous sulfate dissolved in saline (5 mM) was then infused bilaterally into the striatum of these rats using a rat brain stereotaxic apparatus (Stoelting, IL, USA). The stereotaxic coordinates were; 0.8 mm caudal to the bregma, 2.7 mm lateral to the sagital suture and 5 mm ventral to the dura (Paxinos and Watson., 1998). Two microliters of normal saline

was infused into the striatal region, bilaterally, in the sham control animals. The rats were then divided into five groups and treated as described in table 2.4. LD treatment groups also received 2.5 mg/kg of benserazide prepared in saline to prevent the peripheral decarboxylation of LD. Each group contained five rats. The rats were sacrificed on the 8^{th} day by cervical dislocation, followed by decapitation. The striatum of each rat was frozen in liquid nitrogen and stored at -70 $^{\circ}$C.

Table 2.4: Treatment protocol for iron-infused rats

Treatment Group	Intrastriatal Injection (2 µl)	Twice daily drug treatment after intrastriatal infusion
I (control)	Saline	Saline
II	10 nmol of Fe^{2+}	Saline
III	10 nmol of Fe^{2+}	10 mg/kg of LD
IV	10 nmol of Fe^{2+}	2.5 mg/kg of SEL
V	10 nmol of Fe^{2+}	10 mg/kg of LD and 2.5 mg/kg of SEL

2.5.2.4 Preparation of tissue for HPLC analysis

As described in section 2.4.2.4

2.5.2.5 Instrumentation

As described in section 2.2.2.3

2.5.2.6 Chromatographic conditions

As described in section 2.2.2.4

2.5.2.7 Statistical Analysis

As described in section 2.3.2.5

2.5.3 RESULTS

Striatal samples of rats receiving twice daily i.p. injections of LD (10 mg/kg), SEL (2.5 mg/kg) or a combination of these drugs were processed for 6-OHDA. Despite the large quantities of DA in the striatum, as well as the elevation of DA levels in the striatum induced by the administration of LD and SEL, 6-OHDA was not detected in these samples.

In order to increase the chances of hydroxyl radical attack on DA, rats were given an intrastriatal infusion of Fe^{2+} to increase hydroxyl radical formation. This was then followed by twice daily i.p. injections of LD (10 mg/kg), SEL (2.5 mg/kg) or a combination of these drugs. However, despite the large quantities of DA and the likelihood of hydroxyl radical generation, 6-OHDA was still undetectable in these samples. In two striatal samples from rats receiving LD there were small peaks with the same retention time as 6-OHDA. These peaks were however below the limit of quantification.

2.5.4 DISCUSSION

The results of this HPLC-ECD search for 6-OHDA after the acute administration of LD and/or SEL twice daily for seven days revealed no detectable amounts of 6-OHDA in the striatum. An increase in free iron levels in the striatum prior to the administration of LD and/or SEL did not result in detectable levels of 6-OHDA in the striatum. The subsequent oxidative stress and ROS production following the intrastriatal infusion of iron was therefore not sufficient to produce detectable levels of 6-OHDA in the striatum.

Apparently, the formation of 6-OHDA is not responsible for neurodegeneration of DA neurons following LD treatment. These results therefore question the hypothesis that formation of 6-OHDA is responsible for the side effects associated with long term LD treatment. The failure to detect 6-OHDA does not, however exclude the possibility that the concentration of 6-OHDA formed may be below the detection limits of the assay. In addition to this, nucleophilic attack by endogenous compounds with sulfhydryl groups could scavenge oxidized 6-OHDA as fast as it was formed. To test the plausibility of this explanation, a following chapter will investigate the effect of LD and SEL administration on GSH levels, both *in vitro* and *in vivo*.

CHAPTER THREE

PINEAL INDOLE METABOLISM

3.1 INTRODUCTION

Melatonin (MEL) is an endogenous neurohormone synthesized from serotonin (5-HT) in the pineal gland. This is accelerated by the release of norepinepherine (NE) in the pineal gland at night from the sympathetic nerve terminals innervating the gland (Olivieri *et al*., 1990). The NE, via the activation of β-adrenergic receptors causes an increase in the gene expression and enzymatic activity of the pineal enzyme, N-acetyltransferase (NAT) resulting in enhanced conversion of serotonin to N-acetylserotonin (NAS) (Olivieri *et al*., 1990; Reiter, 1991). The amount of NAS synthesized depends on the levels of serotonin accumulated during the day. The conversion of 5-hydroxytryptophan to 5-HT by 5-hydroxytryptophan decarboxylase is highest during the day (light phase). The NAS formed is subsequently converted to melatonin by the enzyme hydroxyindole-O-methyl transferase (HIOMT) (Reiter, 1991). This explains the inverse relationship between the rat pineal concentrations of serotonin and melatonin.

The small size and accessibility of the rat pineal gland allows for easy culturing of the gland in an intact state, without having to use tissue slices. It is well established that the rat pineal gland in organ culture is able to convert radiolabelled [^{14}C] serotonin to [^{14}C] melatonin (Daya *et al*., 1989; Olivieri *et al*., 1990). The [^{14}C] serotonin is also acted upon by the enzyme monoamine oxidase (MAO) to produce a number of [^{14}C] indole metabolites. The indole products derived from 5-HT in the pineal gland are shown in figure 3.1 and include 5-methoxytryptamine (5-MT), melatonin (MEL), N-acetylserotonin (NAS), 5-methoxyindole acetic acid (5-MIAA), 5-hydroxyindole acetic acid (5-HIAA), 5-methoxytryptophol (5-MTOH) and 5-hydroxytryptophol (5-HTOH).

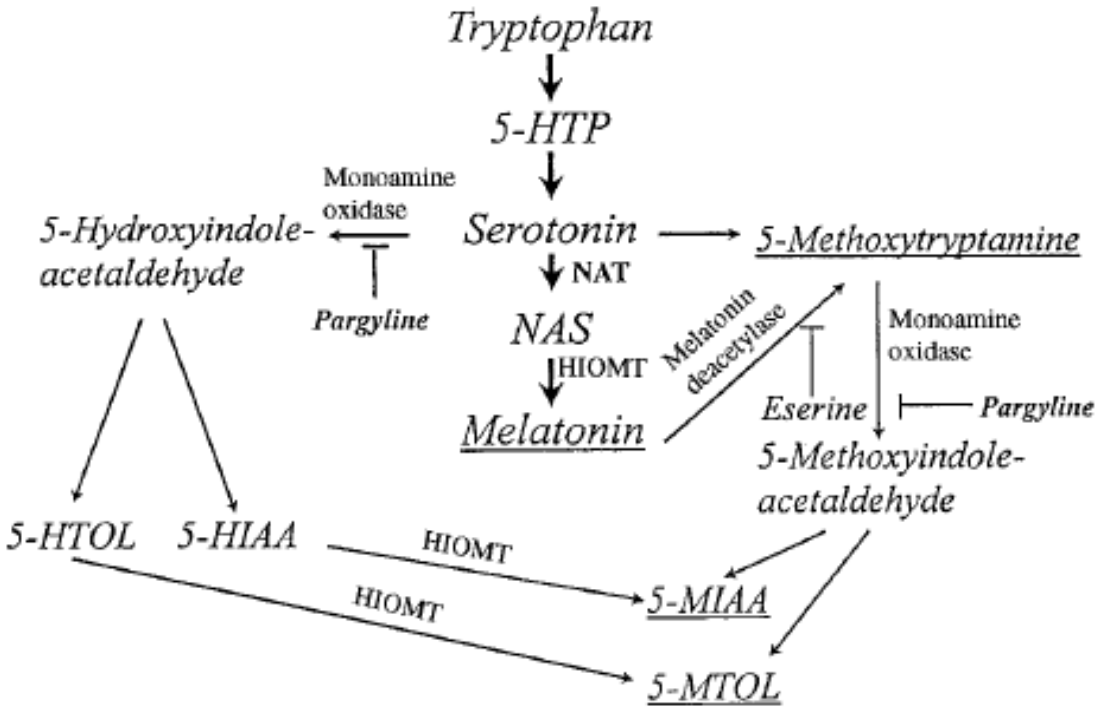


Figure 3.1: A simplified diagram showing the biochemical pathways through which melatonin and the other pineal metabolic products are formed (Yanez and Meissl., 1996).

As much as 95 % of the various [^{14}C] indoles synthesized are secreted into the culture medium. The relative amounts of each indole are similar in the pineal gland and the culture medium (Klein and Rowe, 1970). Thus the metabolites in the culture medium are considered a good reflection of pineal indole metabolism (Klein and Notides, 1969). The [^{14}C] indole products in the culture medium can be separated, identified and quantified by using a bi-dimensional thin layer chromatography (TLC) system, first used by Klein and Notides in 1969. The [^{14}C] indoles are separated using two solvent systems; the first consists of chloroform, methanol and glacial acetic acid (93: 7: 1) and the second solvent system utilizes ethyl acetate only.

3.2 EFFECT OF SELEGILINE ON PINEAL INDOLE METABOLISM

3.2.1 INTRODUCTION

The previous chapter demonstrated that SEL suppresses the formation of 6-OHDA *in vitro* and that it may possibly reduce the endogenous biosynthesis of 6-OHDA. Another possible mechanism by which SEL may protect against the neurotoxicity induced by 6-OHDA is that it may increase the production of MEL and NAS, both of which are powerful anti-oxidants (Wolfler *et al*., 1999; Aguiar *et al*., 2004). The main objective of the present study is to investigate whether SEL, a MAO-B inhibitor alters rat pineal indole biosynthesis. Of particular interest is whether this antiparkinsonian drug affects MEL and NAS synthesis by the rat pineal gland.

3.2.2 MATERIALS AND METHODS

3.2.2.1 Chemicals and reagents

The radiochemical 5-hydroxy-(sidechain-2-^{14}C) tryptamine creatinine sulfate (specific activity 58 mCi/mmol) was purchased from Amersham International, United Kingdom. The compound had a radioactive concentration of 50 µCi/ml. The synthetic indoles 5-HT, 5-MT, 5-HIAA, 5-MIAA, 5-HTOH, 5-MTOH, NAS and MEL were purchased from Sigma Chemical Co., St Louis, USA. BGJb culture medium (Fitton Jackson modification) was purchased from Gibco, Europe and aseptically fortified with antibiotics such as penicillin and streptomycin. The aluminum TLC plates pre-coated with silica gel 60 F_{254} (0.2 mm thickness) were purchased from Merck, Germany. The liquid scintillation cocktail, Packard® Scinillator 299™, was purchased from Packard Instrument Company, Inc., Netherlands. Ethanol, methanol, glacial acetic acid, ethyl

acetate and chloroform were obtained from Saarchem Limited, Krugersdorp, South Africa.

3.2.2.2 Animals

Animals were housed and maintained as described in appendix one.

3.2.2.3 Experimental procedure

3.2.2.3.1 Pineal organ culture

The BGJb culture medium was fortified with µg quantities of sodium benzylpenicillin and streptomycin sulfate prior to the experiment.

***In Vitro* Study**

The intact pineal glands were rapidly excised and placed individually in sterile Kimble glass tubes (borosilicate 10 x 75 mm). Each test tube contained 52 µl of BGJb culture medium. A volume of 10 µl of SEL hydrochloride was then added to the incubation medium to give the required final concentration in a total volume of 70 µl. In the control culture tubes, SEL was replaced with 10 µl of milli-Q water (the vehicle in which the SEL was dissolved). The reaction was then initiated by adding 8 µl of [^{14}C] 5-HT (specific activity 58 mCi/mmol). The atmosphere within the culture tubes was then saturated with carbogen (95 % oxygen: 5 % carbon dioxide), immediately sealed and incubated for a period of 24 hours at 37 $^{\circ}$C in the dark. The incubation was then terminated after 24 hours by removing the pineal glands from the culture medium. The culture medium was then stored at -20 $^{\circ}$C until further analysis by TLC.

***Ex Vivo* Study**

The acute administration of SEL was performed at a dose of 2.5 mg/kg/bd. The vehicle for the delivery of the drug was physiological saline and rats received i.p. injections of SEL (2.5 mg/kg) twice daily for 7 days. On the 8th day the rats were sacrificed by cervical dislocation and the pineals were rapidly transferred to the corresponding pre-labeled Kimble tubes. The final total volume of the culture medium was 60 µl for the *ex vivo* study.

3.2.2.3.2 TLC analysis of indoles

A standard solution of non-radiolabeled indoles was freshly prepared by dissolving 1mg of each of the following indoles: 5-HT, 5-MT, 5-HIAA, 5-HTOH, NAS, MEL, 5-MIAA and 5-MTOH in 1 ml of absolute ethanol containing 1 % m/v of ascorbic acid to keep the oxidation of the indoles to a minimum. This solution was stored at 4 ºC in the dark until use.

A 10 µl aliquot of the sample culture medium was loaded onto a 10 x 10 cm TLC plate, to form a spot no larger than 4-5 mm at a demarcated origin. The spot was then dried under a gentle stream of nitrogen to aid in the drying of the spotted medium and to minimize auto-oxidation of the pineal indoles. Thereafter, a 10 µl aliquot of the standard solution containing all the indoles was also spotted onto the origin, on top of the already spotted culture medium and again dried with nitrogen. The plates were then developed in saturated TLC tanks. The plates were first run twice in the same direction in a mobile phase of chloroform, methanol and glacial acetic acid (93: 7: 1). The solvent front was allowed to move about 9 cm. The TLC plates were then removed from the tank and dried under a stream of nitrogen. The plates were then placed in ethyl acetate at right angles to the first direction and were allowed to develop until the solvent front had moved about 6 cm. The plate was then removed from the TLC tank and dried thoroughly with nitrogen.

The location of each indole was visualized by placing the plate under a UV-Visible light. Each spot was then cut out and placed individually in scintillation vials containing 3 ml of scintillation liquid. The vials were then sealed and shaken vigorously for a period of 20 minutes to facilitate extraction of the indoles into the scintillation liquid. The radioactivity of each [^{14}C] indole was then quantified using a Beckman LS 2800 scintillation counter. The results are expressed as disintegrations per minute (DPM) in 10 µl of the culture medium.

3.2.2.4 Statistical analysis

The data of each [^{14}C] indole in a particular group (n = 5) was pooled to produce a single mean value expressed as DPM/10 µl. Group means (control versus SEL) were then statistically compared by using the Student t-test. The level of significance was accepted at $p < 0.05$.

3.2.3 RESULTS

A typical bi-dimensional chromatogram of the pineal indole metabolites is shown in figure 3.2. The figure shows that excellent separation was achieved for NAS, 5-HIAA, 5-HTOH, MEL, 5-MIAA and 5-MTOH. A disadvantage is that 5-HT and 5-MT both remain at the origin and do not separate from each other. An additional TLC system would have been necessary to achieve separation between these two indoles.

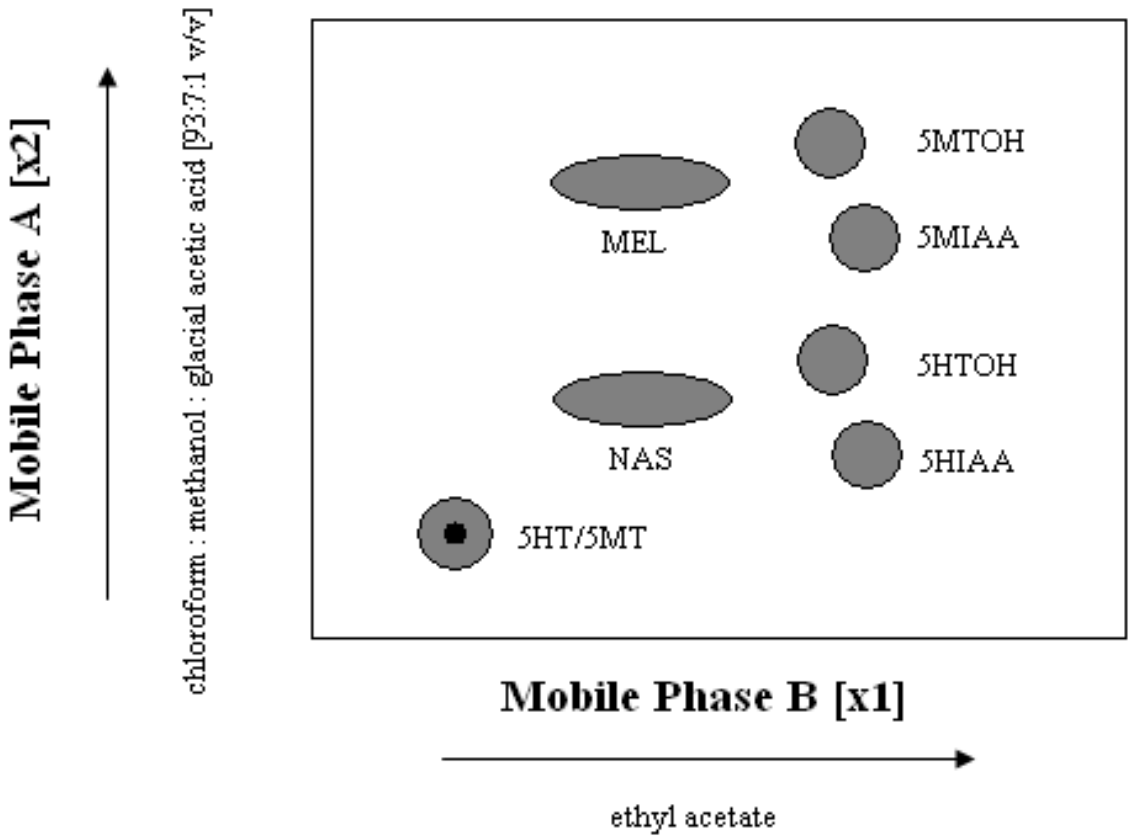


Figure 3.2: A typical bi-dimensional thin layer chromatogram illustrating the direction in which the plate was run and the positions of the pineal indole metabolites (Klein and Notides, 1969).

***In Vitro* Study**

The radioactivity corresponding to each of the metabolites isolated from the culture medium, expressed as disintegrations per minute/10 µl of culture medium is represented in figure 3.3.

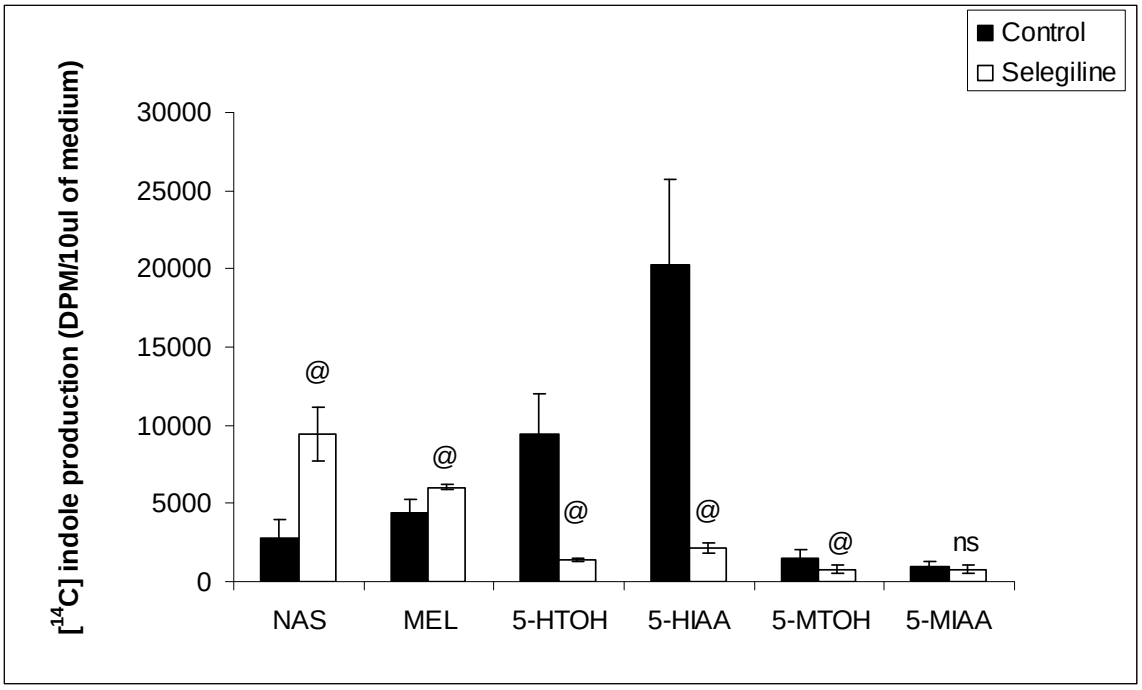


Figure 3.3: The *in vitro* effect of SEL (10 µM) on pineal indole metabolism. Each bar represents the mean ± SD (n = 5). @ ($p < 0.05$) and ns ($p > 0.05$) versus the control.

Figure 3.3 shows that at a concentration of 10 µM, SEL significantly increases the metabolism of serotonin to both NAS and MEL by the rat pineal gland when compared to control values. In addition to an increase in NAS and MEL, SEL also significantly decreases the monoamine oxidase products (5-HIAA, 5-HTOH and 5-MTOH) formed from serotonin. Levels of 5-MIAA formed by the pineal gland are not significantly altered by SEL when compared to control values.

***Ex Vivo* Study**

Figure 3.4 shows that the acute administration of SEL (2.5 mg/kg/bd), results in a significant increase in NAS levels but has no significant effect on MEL or the monoamine oxidase products of 5-HT.

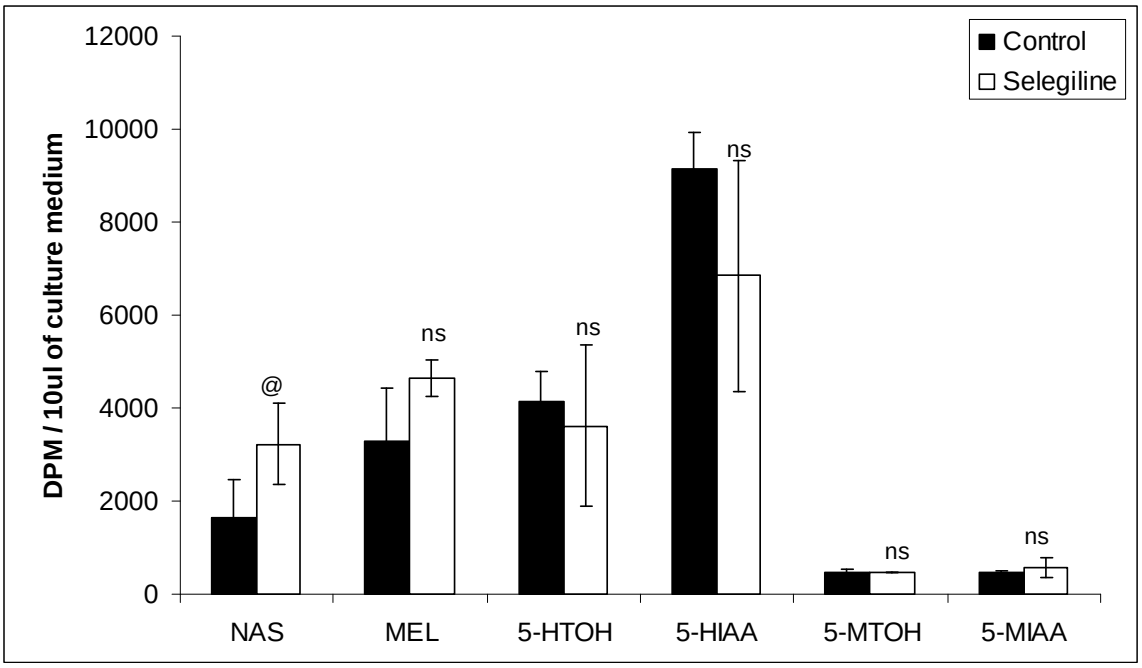


Figure 3.4: The effect of acute administration of SEL (2.5 mg/kg/bd) on pineal indole metabolism. Each bar represents the mean ± SD (n = 5). @ ($p < 0.05$) and ns ($p > 0.05$) versus the control.

3.2.4 DISCUSSION

The results of the *in vitro* experiment indicate that at a concentration of 10 µM, SEL significantly increases melatonin and N-acetylserotonin production by the rat pineal gland and results in a significant decrease in the monoamine oxidase products formed from serotonin. It is tempting to suggest that the SEL induced increase in melatonin production is brought about by elevated concentrations of 5-HT as a result of MAO inhibition. By weakening the MAO-mediated catabolism of 5-HT, the alternative pathway converting serotonin to NAS and MEL is strengthened. However, an increase in the biosynthesis of melatonin in the pineal body following treatment with SEL can not simply be explained by inhibition of MAO-B, because the affinity of MAO-B for 5-HT is low (Nishi *et al*., 2006). Since 5-HT is inactivated primarily by MAO-A (Nishi *et al*., 2006), it is unlikely that SEL, a selective MAO-B inhibitor would result in an elevation

of 5-HT levels in the pineal body. The most likely explanation for the decrease in the monoamine oxidase products of serotonin is that the concentration of SEL used (10 μM) was high enough to cause inhibition of both MAO-B and MAO-A, this would explain the decrease in 5-HTOH, 5-HIAA and 5-MTOH observed. The increase in NAS and MEL production induced by SEL could on the other hand be due to an inhibition of NE degradation or an increase in pineal N-acetyltransferase (NAT) activity. An increase in the activity of NAT would result in an increase in the N-acetylation of 5-HT to NAS, the precursor of melatonin.

The results of the acute administration of SEL (2.5 mg/kg twice daily) on pineal indole metabolism showed a significant increase in NAS levels, however, levels of MEL, 5-HTOH, 5-HIAA, 5-MTOH and 5-MIAA were not significantly altered. The results of this study suggest that the dose of SEL used was not sufficiently high for the drug to lose selectivity for MAO-B and therefore the metabolism of serotonin by MAO-A was unaffected. The rise in NAS levels is most likely due to an increase in pineal NAT activity. NAS has properties similar to that of melatonin, and both indoleamines have protective effects against 6-OHDA induced toxicity (Martin *et al*., 2000; Calvo *et al*., 2001). Recent studies indicate that the antioxidant activity of MEL is actually lower than that of NAS (Oxenkrug, 1999; Wolfler *et al*., 1999; Requintina and Oxenkrug, 2003). An increase in the activity of NAT is brought about by β-adrenergic receptor stimulation (Reiter, 1991). β-adrenergic receptor stimulation induced by SEL administration may be mediated by DA and NE.

The rise in NAS induced by SEL in rats may be another mechanism by which this drug can protect against 6-OHDA formation and toxicity.

In order to gain more insight into the way in which SEL results in increased NAS production in the pineal gland, the following chapter will investigate whether the administration of SEL (2.5 mg/kg/bd) can alter 5-HT, DA and NE levels in the rat striatum and hippocampus.

CHAPTER FOUR

NEUROTRANSMITTER LEVELS

4.1 INTRODUCTION

The homeostasis of DA, 5-HT and NE is maintained in part by the metabolism of these monoamines by MAO, both intracellularly and extracellularly and by COMT, extracellularly (Westerink, 1984). Removal of these amines from the synaptic cleft is also achieved via a reuptake mechanism (Blackburn *et al*., 1967).

DA is a catechol neurotransmitter widely distributed throughout the brain but with most of it found in the striatum (Palkovits and Brownstein, 1983). The two main DA pathways in the brain are the nigrostriatal and mesolimbic pathways. The nigrostriatal system consists of a number of dopaminergic neurons whose cell bodies are located in the *substantia nigra* (SN) and whose axons are projected towards the striatum. The main function of this neuronal system is assumed to be the modulation of voluntary movements. DA is synthesized by the decarboxylation of LD by neuronal L-amino acid decarboxylase (AADC), which in turn is synthesized from tyrosine by the action of tyrosine hydroxylase (Nishi *et al*., 2006). DA is metabolised via two main pathways in the brain by the enzymatic reactions of MAO (both species) and COMT. Approximately 70 – 90 % of DA turnover occurs via the MAO mediated pathway to 3,4-dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA), while 10 – 30% of DA turnover occurs through the COMT mediated pathway extracellularly giving rise to 3-methoxytyramine (3-MT) in the rat striatum (Westerink, 1984; Wood *et al*., 1987). A balance between DA synthesis, catabolism and reuptake maintains DA homeostasis in the brain.

5-HT is an indolealkylamine neurotransmitter synthesized from tryptophan, an essential amino acid. Alterations in brain 5-HT levels have been implicated in a number of neuroendocrine, psychiatric, and neurologic disorders, particularly depression, anxiety,

schizophrenia, obsessive-compulsive disorder and migraines. The mechanism by which 5-HT acts in the brain and interacts with other neurotransmitter systems is not yet clearly understood. There is little data available regarding the influence of SEL and LD on 5-HT synthesis in the rat brain (Nishi et al., 2006).

4.2 EFFECT OF L-DOPA AND SELEGILINE ON BRAIN NEUROTRANSMITTER LEVELS *IN VIVO*

4.2.1 INTRODUCTION

In chapter 2, it was demonstrated that the exogenous administration of LD (10 mg/kg/bd) and SEL (2.5 mg/kg/bd) for 7 days does not result in 6-OHDA formation in the rat striatum, despite the expected increase in striatal DA levels produced by the administration of these drugs. This chapter investigates the extent to which these drugs cause an increase in striatal DA levels. The chapter also investigates whether SEL, at a dose of 2.5 mg/kg/bd for 7 days can affect 5-HT metabolism in the striatum and hippocampus. This will provide further insight into the mechanism by which SEL enhances NAS production by the pineal gland (chapter 3).

In this study, the effect of SEL and LD on brain biogenic amines was determined using an HPLC-ECD method to detect simultaneously DA, HVA, DOPAC, NE, 5-HT and 5-HIAA in the rat striatum and hippocampus following i.p. administration of LD and/or SEL.

4.2.2 MATERIALS AND METHODS

4.2.2.1 Chemicals and reagents

L-3,4-dihydroxyphenylalanine (LD), benserazide hydrochloride, SEL hydrochloride, heptane sulphonic acid (HSA), Ethylenediaminetetraacetic acid (EDTA), triethylamine (TEA), phosphoric acid (PA), perchloric acid ($HClO_4$), dopamine (DA), serotonin (5-HT), dihydroxyphenylacetic acid (DOPAC), homovanillic acid (HVA), 5-hydroxyindoleacetic acid (5-HIAA) and noradrenaline (NE) were purchased from the

Sigma Chemical Corporation, St. Louis, MO, USA. HPLC grade acetonitrile (ACN) was purchased from Saarchem, Johannessburg, South Africa. All other reagents were of the highest quality available.

4.2.2.2 Animals

Animals were housed and maintained as described in appendix one.

4.2.2.3 Drug treatment

Rats were divided into four groups (I – IV), with five rats in each group. Group I served as the control and received saline, Group II received LD, 10 mg/kg/bd and Group III received 2.5 mg/kg/bd of SEL. Group IV received a combination of LD (10 mg/kg/bd) and SEL (2.5 mg/kg/bd). Each animal received a dose volume of 1 µl/g of body weight of LD and/or SEL i.p. twice daily for 7 days. LD treatment groups also received 2.5 mg/kg/bd of benserazide prepared in saline to prevent the peripheral decarboxylation of LD. The animals were sacrificed by cervical dislocation on the 8^{th} day. The striatum and hippocampus of each rat was removed, frozen in liquid nitrogen and stored at -70°C.

4.2.2.4 Preparation of tissue for HPLC analysis

On the day of the experiment, the striatal and hippocampal samples were thawed on ice, weighed and sonicated (50 Hz for 60 s) in ice cold 0.1 M $HClO_4$ containing 0.01 % EDTA using an ultrasonic cell disruptor (1 mg of tissue in 10 µl). The sonicated samples were kept on ice for 30 minutes and centrifuged at 10 000 x g for 10 minutes using a bench top centrifuge. The supernatant was injected directly into the HPLC-ECD system for analysis.

4.2.2.5 Instrumentation

As described in section 2.2.2.3

4.2.2.6 Chromatographic conditions

Separation was achieved using an Ultrasphere C18 IP 80A analytical column (5 µm, 250 x 4.6 mm). The mobile phase consisted of 8.65 mM HSA, 0.27 mM EDTA, 13 % ACN, 0.43 % TEA, 0.32 % PA and made up to 1000 ml with Milli-Q water. The mobile phase was degassed twice using a 0.45 µm membrane filter prior to use. The flow rate was 1 ml/min, and the electrodetection was performed at 0.74 V. Results are expressed as pmol/mg tissue.

4.2.3 RESULTS

Figure 4.1 shows that the acute administration of SEL (2.5 mg/kg/bd) significantly decreases levels of DOPAC and HVA and elevates striatal DA levels, when compared to the control values. The acute administration of LD (10 mg/kg/bd) significantly increases levels of DA, as well as its major acidic metabolite DOPAC, however levels of HVA were not significantly altered in the striatum. In addition to DA, LD administration also resulted in a significant increase in NE levels. Neither SEL nor LD altered striatal levels of 5-HT or its metabolite 5-HIAA. However, when both drugs are administered together, a significant decrease in DOPAC and HVA levels were observed, but striatal DA levels were not significantly altered compared to when LD was administered alone. The administration of both drugs in combination had no effect on striatal levels of 5-HT and 5-HIAA.

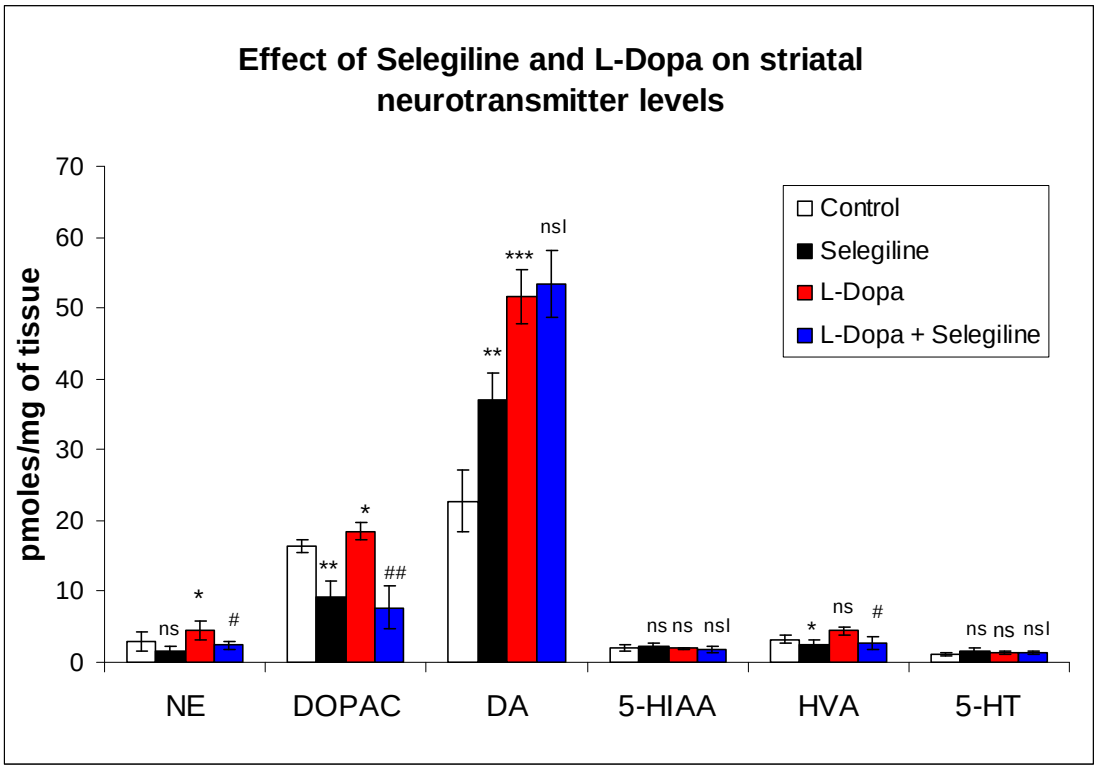


Figure 4.1: The effect of SEL and LD on striatal biogenic amine levels. Each bar represents the mean ± SD (n = 5). ns ($p > 0.05$), * ($p < 0.05$), ** ($p < 0.01$) and *** ($p < 0.001$) compared to the control; nsl ($p > 0.05$), # ($p < 0.05$) and ## ($p < 0.001$) compared to LD treatment groups.

The acute administration of SEL did not alter levels of DA and 5-HT, their metabolites DOPAC and 5-HIAA, or NE levels in the hippocampus when compared to control values (figure 4.2). No values for HVA are reported because no peak was detected for this metabolite in the hippocampus. Figure 4.2 also shows that LD treatment induced a statistically significant increase in NE, DA and DOPAC levels, while levels of 5-HT were unaltered in the hippocampus when compared to the control values. When both drugs were used in combination, no significant differences were observed in the hippocampal samples compared to when LD was administered alone.

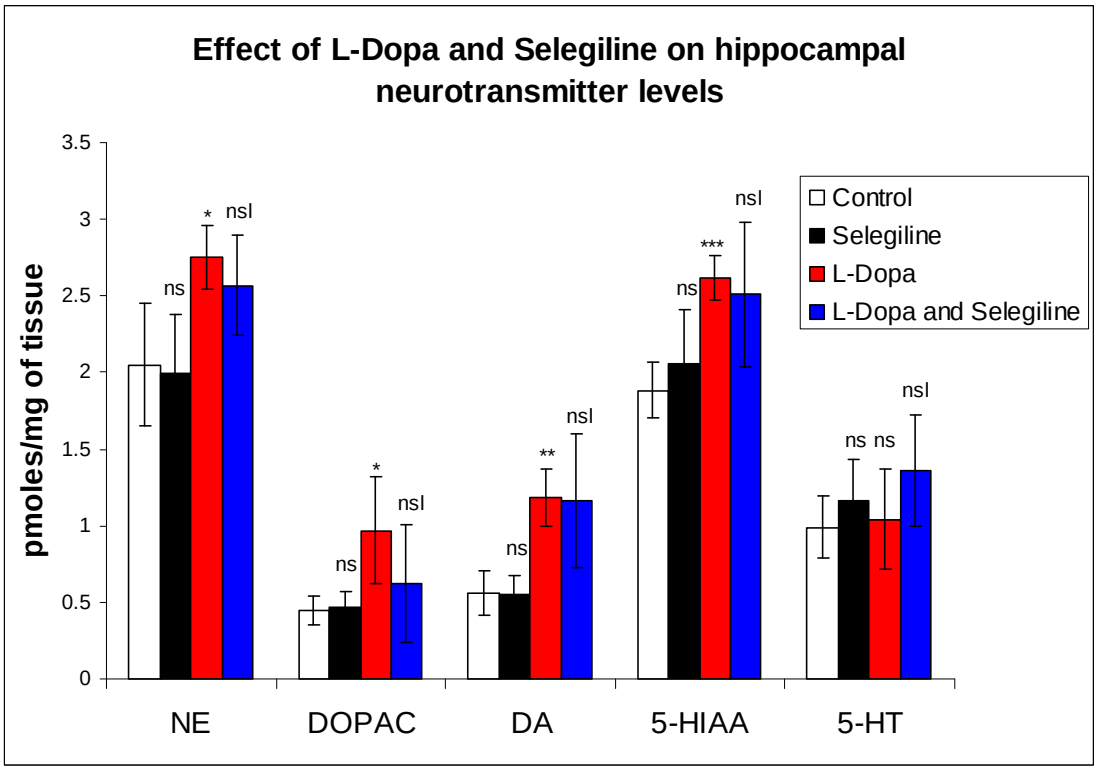


Figure 4.2: The effect of SEL and LD on hippocampal biogenic amine levels. Each bar represents the mean ± SD (n = 5). ns ($p > 0.05$), * ($p < 0.05$), ** ($p < 0.01$) and *** ($p < 0.001$) compared to control; nsl ($p > 0.05$) and # ($p < 0.05$) compared to LD treatment groups.

4.2.4 DISCUSSION

PD is characterized by the degeneration of dopaminergic neurons of the *substantia nigra* and a reduction of DA and its major metabolites DOPAC and HVA in the striatum (Cannazza *et al*., 2005). In the present study, much attention was focused on how LD and SEL affect DA, 5-HT and NE levels in the striatum. The effect of these drugs on DA, 5-HT and NE levels in the hippocampus was also investigated.

The results of the experiment show that the administration of LD elevates striatal and hippocampal DA, DOPAC and NE levels. LD is converted by neuronal aromatic L-amino acid decarboxylase (AADC) into DA (Riobó *et al*., 2002), and therefore the increase in DA levels following LD treatment was to be expected. LD resulted in an approximate

two-fold increase in striatal DA levels. Despite this increase in DA levels, there was still an absence of detectable levels of 6-OHDA in the striatum (chapter 2). Since the catabolism of DA is not inhibited by LD, it follows that increased levels of DA would be accompanied by an increase in the metabolites of DA, such as DOPAC. The increase in NE levels induced by LD could also be due to the increase in DA, since DA can be converted to NE by dopamine-β-hydroxylase (Riobó *et al*., 2002). Both striatal and hippocampal levels of 5-HIAA and 5-HT were unchanged following LD administration.

The elevation of striatal DA levels and the reduction of DOPAC and HVA levels induced by SEL (a selective MAO-B inhibitor) could be explained by inhibition of DA catabolism, resulting in elevated levels of DA and a reduction in DOPAC and HVA. The results of this experiment also show that SEL has no effect on 5-HT or 5-HIAA levels after intraperitoneal administration. This can be explained by the fact that DA is a substrate for both forms of MAO, whereas 5-HT is a selective substrate for MAO-A (Youdim and Tipton, 2002). The concentration of SEL used in this study may not be fully specific for MAO-B and could have produced a small inhibition of MAO-A, however according to the findings of Green and Youdim, (1975) and Green *et al*. (1977), it is necessary to inhibit at least 80 % of the enzyme in order to achieve any significant increase in 5-HT levels. When used on its own, SEL had no effect on DA, 5-HT, their metabolites DOPAC, HVA and 5-HIAA or NE levels in the hippocampus. This may be due to the fact that the hippocampus is a region of the brain with a high concentration of MAO-A. The inhibition of MOA-B may not have affected DA levels in this region of the brain because of the more or less equal affinity of MAO-A and MAO-B for DA and the presence of high concentrations of MAO-A in these dopaminergic neurons (Kondoh *et al*., 1994). On the other hand, concentrations of MAO-A in the striatum are relatively low (Kondoh *et al*., 1994).

When both SEL and LD are administered together, a significant reduction in striatal DOPAC and HVA were detected compared to when LD was used alone. However, despite the decrease in DOPAC and HVA, it is interesting to note that the complementation of LD treatment with SEL did not result in a statistically significant

increase in striatal DA levels. In the case of the hippocampus, combined treatment did not alter levels of any of the neurotransmitters measured compared to when LD was administered alone.

Since SEL treatment did not affect 5-HT levels in the striatum and hippocampus, it is unlikely that it would have any effect on the metabolism of 5-HT in the rat pineal gland. The elevation of NAS in the pineal gland following the administration of SEL (2.5 mg/kg/bd) observed in the previous chapter is therefore more likely due to an increase in NAT activity than inhibition of 5-HT metabolism.

CHAPTER FIVE

SUPEROXIDE ANION FORMATION

5.1 INTRODUCTION

The brain is particularly susceptible to free radical damage as a result of its high consumption of total body oxygen and its relatively low concentrations of antioxidant enzymes (Coyle and Puttfarcken, 1993). The physicochemical properties of oxygen require that it gain electrons one at a time. If one electron is added to the outer orbital of oxygen, the superoxide radical anion ($O_2^{\bullet -}$) is formed (Nohl *et al*., 2005).

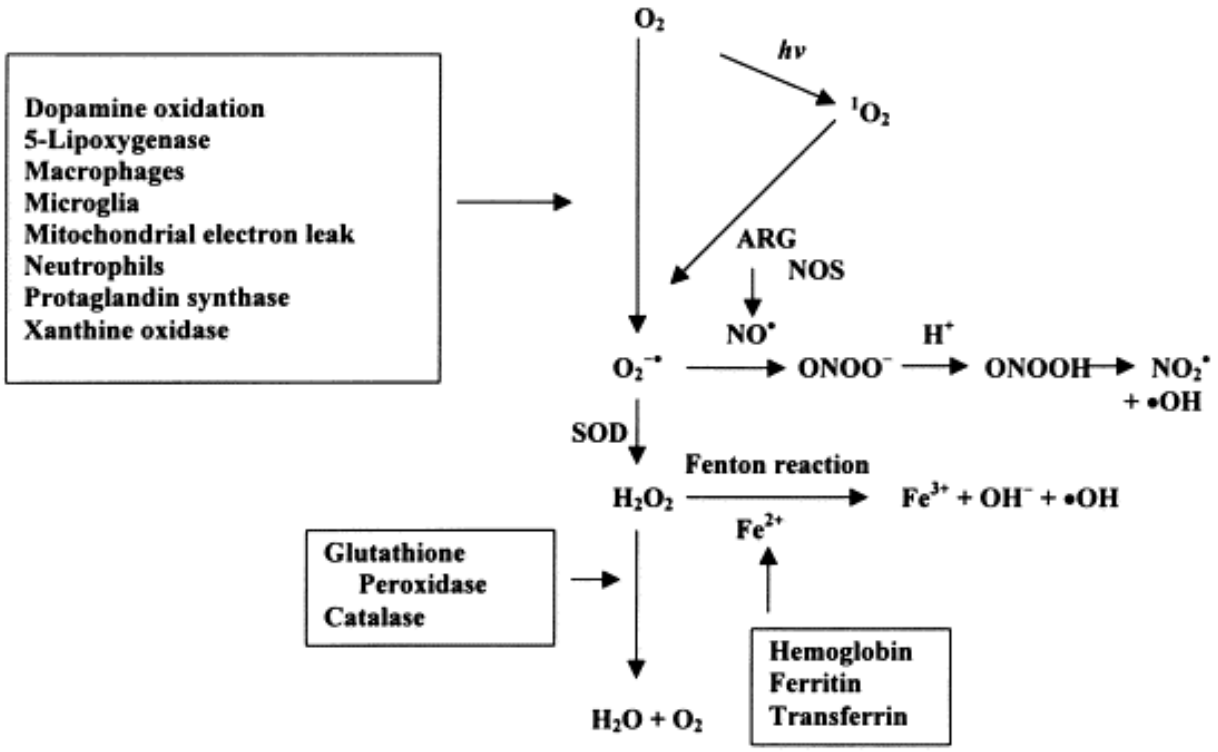


Figure 5.1: A summary of the multiple by-products formed by the partial reduction of oxygen (Reiter, 1998)

Superoxide Anion Formation

The generation of $O_2^{\bullet-}$ is often the start of the oxidative stress cascade and is the precursor to H_2O_2 as well as the more reactive and damaging •OH (figure 5.1). Once formed, $O_2^{\bullet-}$ is inactivated by a family of enzymes, the superoxide dismutates (SOD) to H_2O_2 and further by catalase and glutathione peroxidase to water (Fridovich, 1989). The H_2O_2 can be converted to •OH via the Fenton or Haber-Weiss reaction.

$O_2^{\bullet-}$ is formed *in vivo* in a number of ways (figure 5.1). The non-enzymatic auto-oxidation of 6-OHDA, DA and other catecholamine molecules results in the generation of $O_2^{\bullet-}$ (Graham *et al*., 1978; Klegeris *et al*., 1994). Superoxide can also be produced from lipoxygenases, cyclooxygenases, xanthine oxidase and various flavin oxidases (Cross and Jones 1991; Coyle and Puttfarcken, 1993). Even though there are numerous sources of $O_2^{\bullet-}$, the mitochondrion is considered to be the main site of $O_2^{\bullet-}$ production in mammalian cells through electron leakage from the electron transport chain onto oxygen. In a normally functioning mammalian electron transport chain, electrons are transferred from NADH to the oxidized form of coenzyme Q (UQ), to yield the reduced form of coenzyme Q (UQH_2). UQH_2 then transfers electrons to cytochrome c and is converted to UQ, passing first through the semiquinone anion species ($UQ^{\bullet-}$). Finally reduced cytochrome c is re-oxidized by cytochrome c oxidase (Kowaltowski and Vercesi, 1998). One oxygen molecule is reduced to two molecules of water for every four electrons taken in by the complex (figure 5.2).

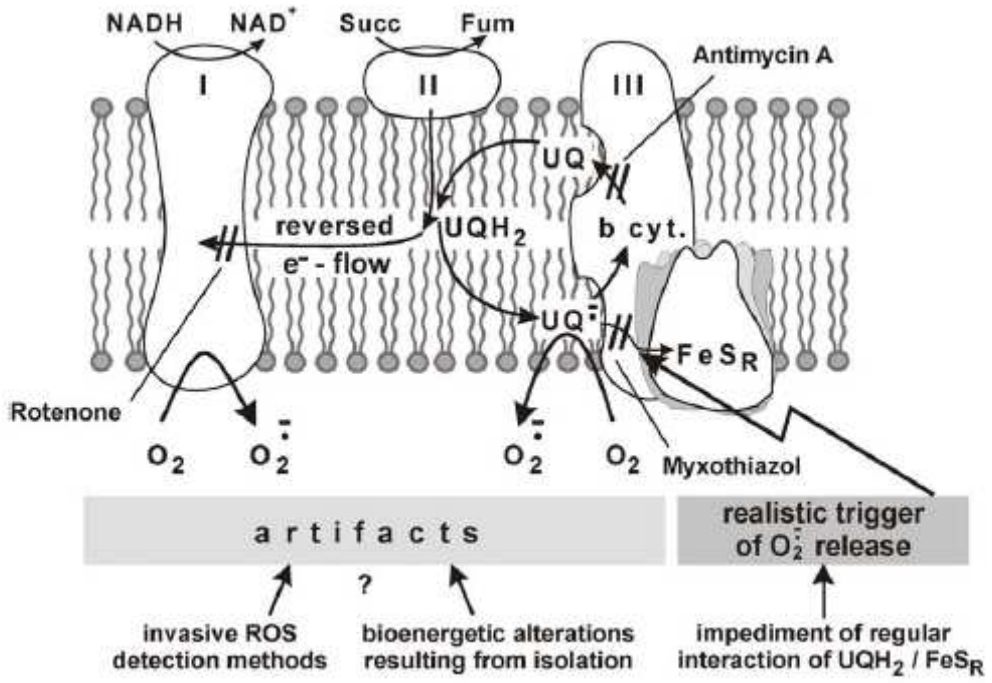


Figure 5.2: The formation of $O_2^{\bullet-}$ from the mitochondrial electron transport chain (Nohl *et al*., 2005).

The main sites of electron leakage seem to be the NADH-coenzyme Q reductase complex (Turrens and Boveris, 1980), and the reduced forms of coenzyme Q itself (Cadenas *et al*, 1977).

The auto-oxidation of DA and 6-OHDA could enhance the generation of $O_2^{\bullet-}$. In addition to this both DA and 6-OHDA are able to interact with and inhibit mitochondrial complex I, II and IV (Glinka and Youdim, 1995; Khan *et al*., 2005; Mazzio *et al*., 2004). This could obstruct the flow of electrons through the electron transport chain and enhance $O_2^{\bullet-}$ production by electron leakage to oxygen. Cytochromes are heme proteins which accept one electron at a time per molecule by allowing Fe^{3+} at the centre of the heme ring to be reduced to Fe^{2+}. Studies have shown that 6-OHDA and to a lesser extent, DA is able to reduce and release iron as Fe^{2+}, from the iron storage protein ferritin (Montinero and Winterbourne, 1989; Linert *et al*., 1996; Babincová and Babinec, 2005). If 6-OHDA and DA reduce Fe^{3+} to Fe^{2+} in the heme core of CYT-C (maintaining it in its reduced form),

this could possibly obstruct electron flow through complexes II and III and increase $O_2^{\bullet-}$ formation.

The previous chapter shows that intraperitoneal administration of LD results in a significant elevation of striatal DA levels. The main aim of this study is to investigate the effect of DA and 6-OHDA on $O_2^{\bullet-}$ formation in rat brain homogenate *in vitro* and compare this to the effect that LD administration has on $O_2^{\bullet-}$ production *in vivo.* In each study SEL was used to examine its ability to trap $O_2^{\bullet-}$ and inhibit any rise in $O_2^{\bullet-}$ produced by DA, 6-OHDA or intraperitoneal LD treatment.

5.2 EFFECT OF DOPAMINE AND 6-HYDROXYDOPAMINE ON SUPEROXIDE ANION FORMATION IN RAT BRAIN HOMOGENATE *IN VITRO*

5.2.1 INTRODUCTION

Several lines of evidence suggest that mitochondrial dysfunction is involved in the dopaminergic neuronal death associated with PD (Jenner, 1993; Greenamyre *et al*, 1999). Analysis of post mortem brain tissue has indicated a decrease in the activity of mitochondrial NADH dehydrogenase (complex I) in the *substantia nigra* of PD patients (Schapira *et al*., 1990). It is generally accepted that oxidative damage mediated by DA oxidation products contributes to the mitochondrial defects in PD (Cohen *et al*., 1997; Berman and Hastings, 1999; Gluck *et al*., 2002). Another contributing factor could be the endogenous formation of 6-OHDA, a toxic product of •OH attack on DA.

The auto-oxidation of DA and 6-OHDA generate substantial amounts of $O_2^{\bullet-}$, H_2O_2 and •OH. A number of studies have also shown that DA and 6-OHDA have the ability to obstruct electron flow along the electron transport chain by inhibiting mitochondrial complex I, II and IV (Glinka and Youdim, 1995; Khan *et al*., 2005; Mazzio *et al*., 2004). This study therefore investigates the potential of DA and 6-OHDA to cause $O_2^{\bullet-}$ production in rat brain homogenate by using the nitroblue tetrazolium (NBT) assay. This method is generally accepted as a reliable method of assaying for $O_2^{\bullet-}$ and possibly other free radicals (Das *et al*., 1990; Sagar *et al*., 1992). The assay involves the reduction of the yellow NBT dye by $O_2^{\bullet-}$ to the blue insoluble NBD, which can be extracted with glacial acetic acid. The formation of NBD can then be measured at 560 nm using a UV spectrophotometer.

5.2.2 MATERIALS AND METHODS

5.2.2.1 Chemicals and reagents

DA hydrochloride, 6-OHDA hydrobromide, nitroblue diformazan (NBD) and nitroblue tetrazolium (NBT) were purchased from Sigma Chemical Corporation, St Louis, MO, U.S.A. Glacial acetic acid and ethanol were purchased from Saarchem, Johannesburg, South Africa. All other reagents were of the highest quality available from commercial sources.

A 0.1 % solution of NBT was prepared by dissolving the NBT in a few drops of absolute ethanol before making it up to the required volume with Milli-Q water.

5.2.2.2 Preparation of the standard curve

NBD was used to prepare the standard curve. A series of reaction tubes, each containing appropriate concentrations of NBD (at 50 µM intervals) dissolved in glacial acetic acid was prepared. A calibration curve (appendix three) was then generated by measuring the absorbance at 560 nm using a Shimadzu UV-160A UV-visible recording spectrophotometer.

5.2.2.3 Animals

Animals were housed and maintained as described in appendix one.

5.2.2.4 Brain removal

Rats were sacrificed by cervical dislocation, followed by decapitation, and the brains were rapidly removed for use in experiments as described in appendix two. The brains were either used immediately or stored at -70 ºC until needed.

5.2.2.5 Homogenate preparation

Each brain was weighed and homogenized (10 % m/v) in ice cold 0.1 M PBS, pH 7.4 in a glass teflon homogenizer. This is necessary to prevent lysosomal damage to the tissue. The homogenate was then used immediately for the assay.

5.2.2.6 Nitroblue tetrazolium assay

A modified method by Das *et al*. (1990) was used for this assay.

Rat brain homogenate (1 ml) containing various concentrations of either DA or 6-OHDA (0. 0.05, 0.1, 0.15, 0.2, 0.25 mM) were incubated with 0.4 ml of 0.1 % NBT in an oscillating water bath for 1 hour at 37 ºC. Termination of the assay and extraction of the reduced NBT was carried out by centrifuging the samples at 2000 x g for 10 minutes and then re-suspending the pellet with 2 ml of glacial acetic acid. The absorbance of the glacial acetic acid fraction was then measured at 560 nm and converted to nmol of diformazan using the standard curve generated from NBD. Final results are expressed as nmol of NBD/mg of tissue.

5.2.2.7 Statistical analysis

As described in section 2.3.2.5

5.2.3 RESULTS

Figure 5.3 shows that exposure of whole rat brain homogenate to DA and 6-OHDA results in a concentration dependent increase in $O_2^{\bullet-}$ formation. Results in figure 5.3 represent the mean ± SD of five determinations. The 0.25 mM concentration of DA resulted in a ± 50 % increase in $O_2^{\bullet-}$ production and the 0.25 mM concentration of 6-OHDA resulted in a ± 66 % increase in $O_2^{\bullet-}$ production compared to the control values.

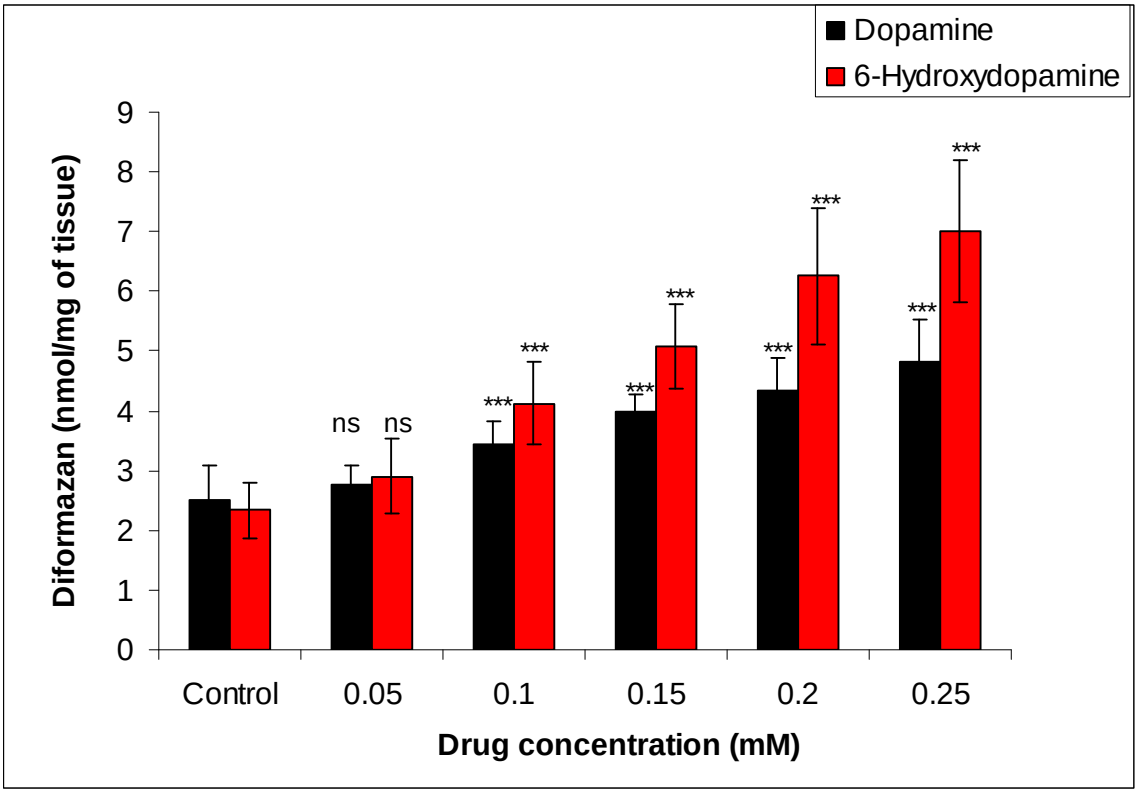


Figure 5.3: Concentration dependent effect of DA and 6-OHDA on $O_2^{\bullet-}$ formation in whole rat brain homogenates. Each bar represents the mean ± SD (n = 5). ns ($p > 0.05$) and *** ($p < 0.001$) compared to the control group.

5.2.4 DISCUSSION

The results of this study demonstrate that DA and 6-OHDA result in a rapid and concentration dependent increase in $O_2^{\bullet-}$ production in whole rat brain homogenate.

The auto-oxidation of DA and 6-OHDA is known to contribute to the production of $O_2^{\bullet-}$, and other reactive oxygen species (ROS) that are capable of destroying the structural and functional apparatus of neurons in the SN (Blum *et al*., 2001). 6-OHDA has a faster rate of auto-oxidation than DA and therefore has the ability to generate greater amounts of $O_2^{\bullet-}$ in a given time (Riobó *et al*., 2002), this is in agreement with the results obtained in this study. However, the auto-oxidation of these compounds is unlikely to account for all the $O_2^{\bullet-}$ produced because when the experiment was repeated in the absence of rat brain homogenate, DA and 6-OHDA were not able to convert the yellow NBT dye to produce a blue NBD precipitate during the 1 hour incubation. In contrast to this, when DA and 6-OHDA were incubated at 37 ºC for 1 hour in the presence of rat brain homogenate, a dark bluish purple precipitate (NBD) was obtained.

Another mechanism by which DA and 6-OHDA may contribute to the production of $O_2^{\bullet-}$ is that these agents may interfere with the mitochondrial electron transport chain. Studies have shown that DA and 6-OHDA are capable of inhibiting mitochondrial complexes I, II and IV (Glinka and Youdim, 1995; Khan *et al*., 2005; Mazzio *et al*., 2004). This could obstruct the flow of electrons through the electron transport chain and enhance $O_2^{\bullet-}$ production by increased electron leakage onto oxygen.

Previous studies have shown that 6-OHDA can reduce Fe^{3+} to Fe^{2+}, initiating the release of free iron from its binding site in ferritin, the main iron transport protein (Montinerio and Winterbourne, 1989; Linert *et al*., 1996; Babincova and Babinec, 2005). A later chapter will also try to confirm the results of these studies. It has also been demonstrated that the release of iron from ferritin can alter mitochondrial calcium homeostasis (Frei and Richter, 1968). An increase in intracellular calcium can initiate a cascade of events that also lead to ROS generation (Southgate and Daya, 1999). The reductive properties of

6-OHDA on cytochrome c (CYT-C) may also be contributing to impaired mitochondrial function and enhanced $O_2^{\bullet-}$ generation. The reduction of Fe^{3+} to Fe^{2+} in the heme core of CYT-C could lead to the stabilization of the reduced form of cytochrome c (CYT-C-RED). The loss of available CYT-C-OX would prevent electrons received from complex II from being transferred to complex IV (Mazzio *et al*., 2004). This obstruction of the flow of electrons along the electron transport chain could also result in increased $O_2^{\bullet-}$ production. DA may affect the oxidation state of cytochromes in a similar manner to 6-OHDA, however, it is a much weaker reducing agent than is 6-OHDA.

5.3 EFFECT OF SELEGILINE ON DOPAMINE AND 6-HYDROXYDOPAMINE INDUCED SUPEROXIDE ANION FORMATION IN RAT BRAIN HOMOGENATE *IN VITRO*

5.3.1 INTRODUCTION

The auto-oxidation and MAO mediated metabolism of DA and 6-OHDA leads to the production of several deleterious products such as H_2O_2, oxygen radicals and reactive quinones, which are capable of causing mitochondrial dysfunction (Khan *et al*., 2005). The generation of H_2O_2 and other oxygen radicals may play a role in the DA and 6-OHDA mediated inactivation of mitochondrial complex I, II and IV. As mentioned previously, the inhibition of these complexes in the electron transport chain may enhance the generation of $O_2^{\bullet-}$. Since mitochondrial MAO catalyses the oxidative deamination of DA and 6-OHDA (Cohen and Werner, 1994), with concomitant generation of free radicals (figure 5.4) this study sought to investigate the effect of SEL on the production of $O_2^{\bullet-}$ induced by DA and 6-OHDA.

Besides inhibiting MAO-B, evidence has also been presented that SEL may protect the CNS by direct free radical scavenging activity. Thomas *et al*. (1997) reported that SEL had the ability to trap both hydroxyl and peroxyl radicals. The direct scavenging of $O_2^{\bullet-}$ by SEL may also reduce $O_2^{\bullet-}$ production induced by DA and 6-OHDA.

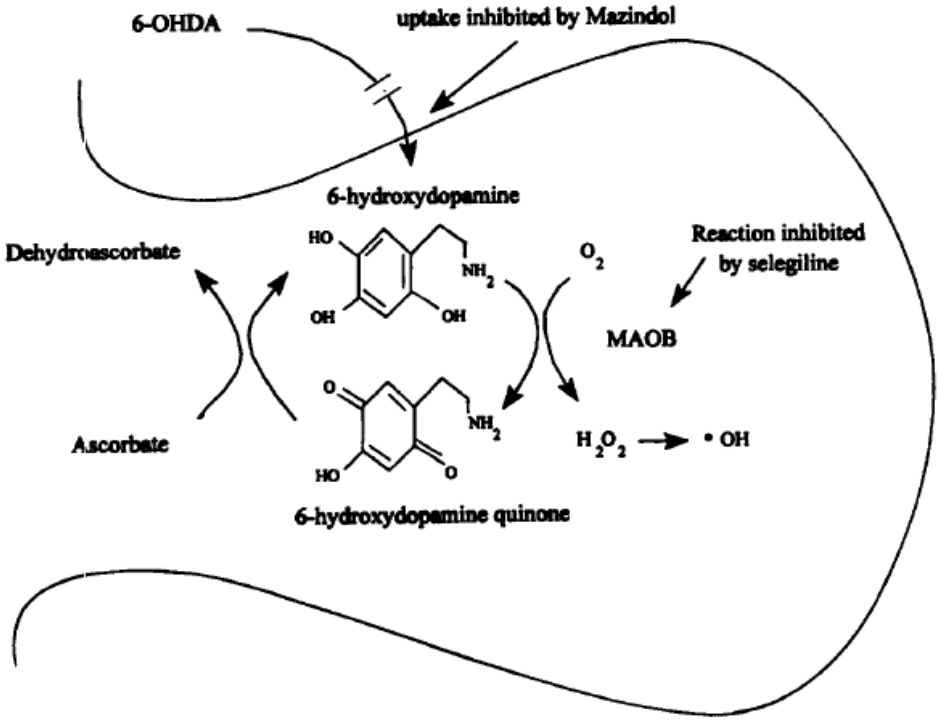


Figure 5.4: Metabolism of 6-OHDA by MAO-B, generating H_2O_2 and •OH and its blockade by SEL (Ebadi *et al*., 1996).

The main aim of this study is to investigate the effect of SEL on the production of $O_2^{\bullet-}$ induced by DA and 6-OHDA observed in the previous study.

5.3.2 MATERIALS AND METHODS

5.3.2.1 Chemicals and reagents

SEL hydrochloride was purchased from Sigma Chemical Corporation, St Louis, MO, U.S.A. All other chemicals and reagents were purchased as described in section 5.2.2.1.

5.3.2.2 Preparation of the standard curve

As described in section 5.2.2.2

5.3.2.3 Animals

Animals were housed and maintained as described in appendix one.

5.3.2.4 Brain removal

As described in section 5.2.2.4

5.3.2.5 Homogenate preparation

As described in section 5.2.2.5

5.3.2.6 Nitroblue tetrazolium assay

A modified method by Das *et al*. (1990) was used for this assay.

The experiment was conducted as described in section 5.2.2.6. The rat brain homogenate (1 ml) was incubated at 37 ºC for one hour, with the highest concentration of either DA or 6-OHDA used in the previous experiment (0.25 mM) alone or in combination with increasing concentrations of SEL. Following this, the NBT assay was performed.

5.3.2.7 Statistical analysis

As described in section 2.3.2.5

5.3.3 RESULTS

As shown in figure 5.5, the treatment of rat brain homogenate with 0.25 mM of DA results in a significant increase in $O_2^{\bullet-}$ production. Figure 5.5 also shows that the treatment of the rat brain homogenate with SEL (0.25, 0.5, 0.75 and 1 mM) reduces the DA induced rise in $O_2^{\bullet-}$ back to control levels. All concentrations of SEL abolished the DA induced rise in $O_2^{\bullet-}$ formation.

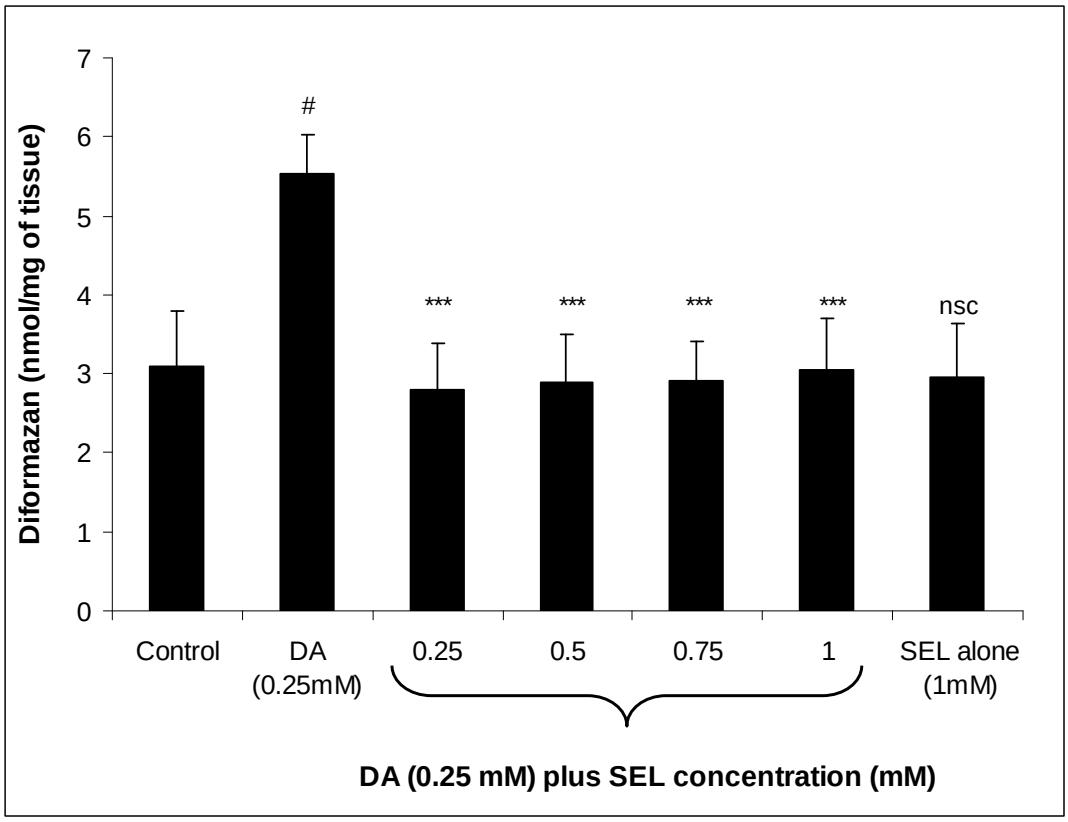


Figure 5.5: Effect of SEL on DA induced $O_2^{\bullet-}$ formation in whole rat brain homogenate. Each bar represents the mean ± SD (n=5). # ($p < 0.001$) and nsc ($p > 0.05$) compared to control values; *** ($p < 0.001$) compared to 0.25 mM of DA.

From figure 5.6, it is evident that exposure of whole rat brain homogenate to 0.25 mM of 6-OHDA results in a significant increase in $O_2^{\bullet-}$ production compared to the control group. Figure 5.6 also shows that treatment of the homogenate with increasing concentrations of SEL (0.25, 0.5, 0.75 and 1 mM) has no effect on the 6-OHDA induced increase in $O_2^{\bullet-}$ production. SEL alone (1 mM) has no effect on $O_2^{\bullet-}$ production when compared to control values.

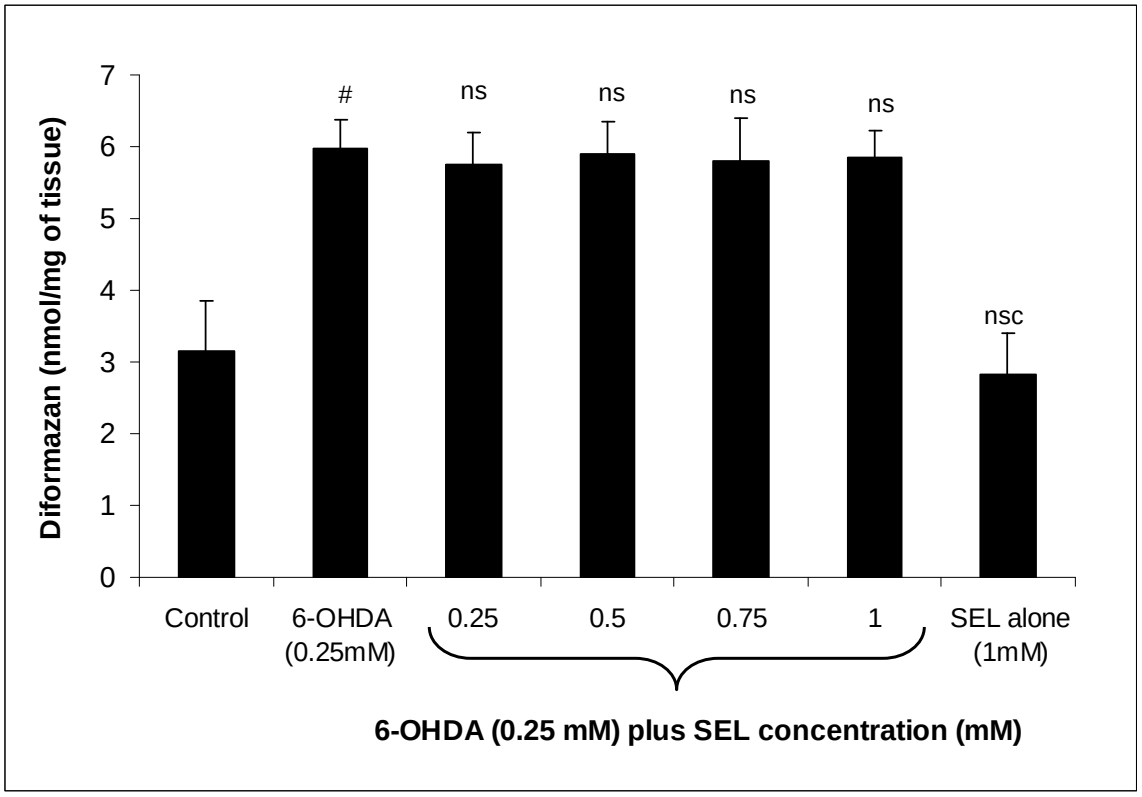


Figure 5.6: Effect of SEL on 6-OHDA induced $O_2^{\bullet-}$ formation in whole rat brain homogenate. Each bar represents the mean ± SD (n=5). # ($p < 0.001$) and nsc ($p > 0.05$) compared to control values; ns ($p > 0.05$) compared to 0.25 mM of 6-OHDA.

5.3.4 DISCUSSION

The results of this study demonstrate that SEL abolishes the rise in $O_2^{\bullet-}$ production in rat brain homogenate induced by DA, but has no effect on the increased $O_2^{\bullet-}$ production induced by 6-OHDA.

The main catabolic pathway for DA in the brain is metabolism by MAO to DOPAC and HVA. This study shows that SEL is able to inhibit the formation of $O_2^{\bullet-}$ by DA. This may be partly because DOPAC auto-oxidizes at a faster rate than DA (Ito *et al*., 1988). The auto-oxidation of DOPAC would therefore be expected to produce larger amounts of $O_2^{\bullet-}$ in a given time than DA. In addition to this DOPAC quinone (DOPAC-Q) may have a higher thiol (-SH) reactivity than DA-quinone (DA-Q) (Khan *et al*., 2005). The total protein content for the mitochondrial membrane, both the inner and outer membrane, varies between 60 % and 65 %. The inner membrane protein content is as high as 75 % (Kowaltowski and Vercesi, 1998). Hydroxyl radicals, as well as the quinone oxidation products of DA and DOPAC are capable of promoting the oxidation of protein cysteine (contains thiol groups) (Rotman *et al*., 1976; Ito *et al*., 1988; Basma *et al*., 1995). Thiol cross-linkage is associated with mitochondrial dysfunction (Fagian *et al*., 1990) and may also be a contributing factor in the inhibition of mitochondrial complex I and IV by DA. It is, therefore, entirely possible that the protection offered by SEL against the DA induced rise in $O_2^{\bullet-}$ formation is at least in part related to the fact that it could prevent the formation of DOPAC quinones and other oxygen radicals formed during the auto-oxidation of DOPAC by inhibiting the metabolism of DA by MAO-B. SEL would not have however prevented the auto-oxidation of DA and the formation of DA-Q. It is possible that SEL prevents the increase in $O_2^{\bullet-}$ production from the auto-oxidation of DA by direct scavenging of the $O_2^{\bullet-}$ radical.

SEL was unable to attenuate the rise in $O_2^{\bullet-}$ induced by 6-OHDA. This may be partly due to the fact that the rate of auto-oxidation of 6-OHDA is greater than that of DA and DOPAC (Cohen and Heikkila, 1974) and 6-OHDA could therefore generate substantial amounts of $O_2^{\bullet-}$. 6-OHDA is also more readily converted to 6-OHDA quinone (6-OHDA-Q) than DA is to DA-Q (Li and Christensen, 1994). A later study will investigate the thiol reactivity of 6-OHDA-Q compared to that of DA-Q by examining the effect of these molecules on GSH levels. In addition to this 6-OHDA is a powerful reducing agent and may have blocked the flow of electrons along the mitochondrial electron transport chain by maintaining components of the chain in their reduced state (figure 5.7) , thereby preventing normal oxidation-reduction events (Glinka and Youdim,

1995; Mazzio *et al*., 2004). As mentioned previously this may have enhanced the production of $O_2^{\bullet-}$ by increasing the leakage of electrons out of the electron transport chain onto oxygen.

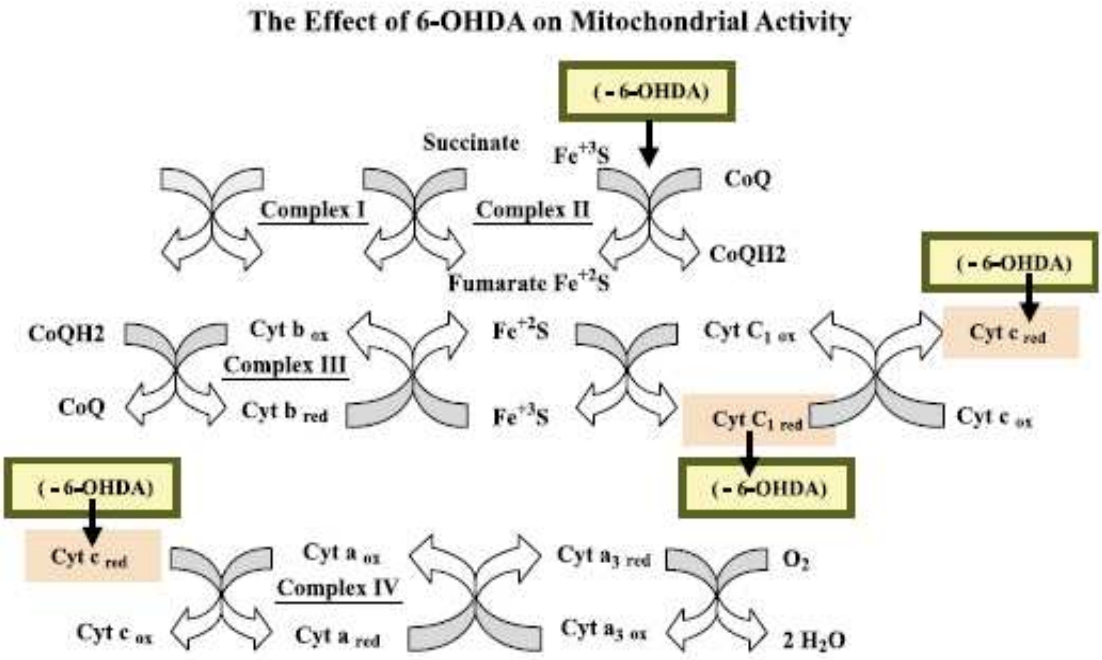


Figure 5.7: Theoretical diagram describing impairment of mitochondrial function by 6-OHDA through alteration of the oxidation state of CYT-C (Mazzio *et al*., 2004).

The following experiment will investigate the effect of intraperitoneal LD and/or SEL administration (which elevate DA levels) on the formation of $O_2^{\bullet-}$ in the rat striatum.

5.4 EFFECT OF L-DOPA AND SELEGILINE TREATMENT ON STRIATAL SUPEROXIDE ANION FORMATION *IN VIVO*

5.4.1 INTRODUCTION

The previous experiments demonstrate that DA and 6-OHDA can significantly increase $O_2^{\bullet-}$ production in whole rat brain homogenate *in vitro*. The systemic administration of LD increases the concentration and turnover of DA in the striatum and it is therefore possible that the administration of LD results in an increase in endogenous $O_2^{\bullet-}$ formation in the striatum. The formation of $O_2^{\bullet-}$ might contribute to neural degeneration and the progression of PD by leading to the formation of the highly reactive and tissue damaging hydroxyl radical. Experimental studies have also shown that LD alters cellular energy metabolism, probably by inducing oxidative damage to the mitochondrial respiratory chain (Przedborski *et al*., 1993; Pardo *et al*., 1995b).

The purpose of this study is to determine whether the acute administration of LD to male Wistar rats results in an increased $O_2^{\bullet-}$ production in the striatum. In addition, the study also investigates the effect of SEL administration on $O_2^{\bullet-}$ production *in vivo*.

5.4.2 MATERIALS AND METHODS

5.4.2.1 Chemicals and reagents

L-3,4-dihydroxyphenylalanine (LD) was purchased from Sigma Chemical Corporation, St Louis, MO, U.S.A. All other chemicals and reagents were purchased as described in section 5.2.2.1.

5.4.2.2 Preparation of the standard curve

As described in section 5.2.2.2

5.4.2.3 Animals

Animals were housed and maintained as described in appendix one.

5.4.2.4 Drug treatment

This was performed as described in section 4.2.2.3

5.4.2.5 Homogenate preparation

On the day of the experiment, striatal samples were thawed on ice, weighed and homogenized (5 % m/v) in ice cold 0.1 M PBS, pH 7.4 in a glass teflon homogenizer. The homogenate was then used immediately for the assay.

5.4.2.6 Nitroblue tetrazolium assay

A modified method by Das *et al*. (1990) was used for this assay.

The assay was conducted as described in section 5.2.2.6. The only difference was that due to the small size and mass of the striatum a smaller volume of striatal homogenate (0.50 ml) was used for the assay. In addition to this no exogenous DA or SEL was added to the homogenate.

5.4.2.7 Statistical analysis

As described in section 2.3.2.5.

5.4.3 RESULTS

Figure 5.8 shows that the acute administration of LD results in a significant rise in striatal $O_2^{\bullet-}$ formation ($p < 0.01$) compared to the control values. However, the co-treatment of rats with SEL in combination with LD was able to reduce the LD enhanced generation of $O_2^{\bullet-}$. Furthermore, the administration of SEL alone was able to decrease the generation of $O_2^{\bullet-}$ below control values ($p < 0.001$).

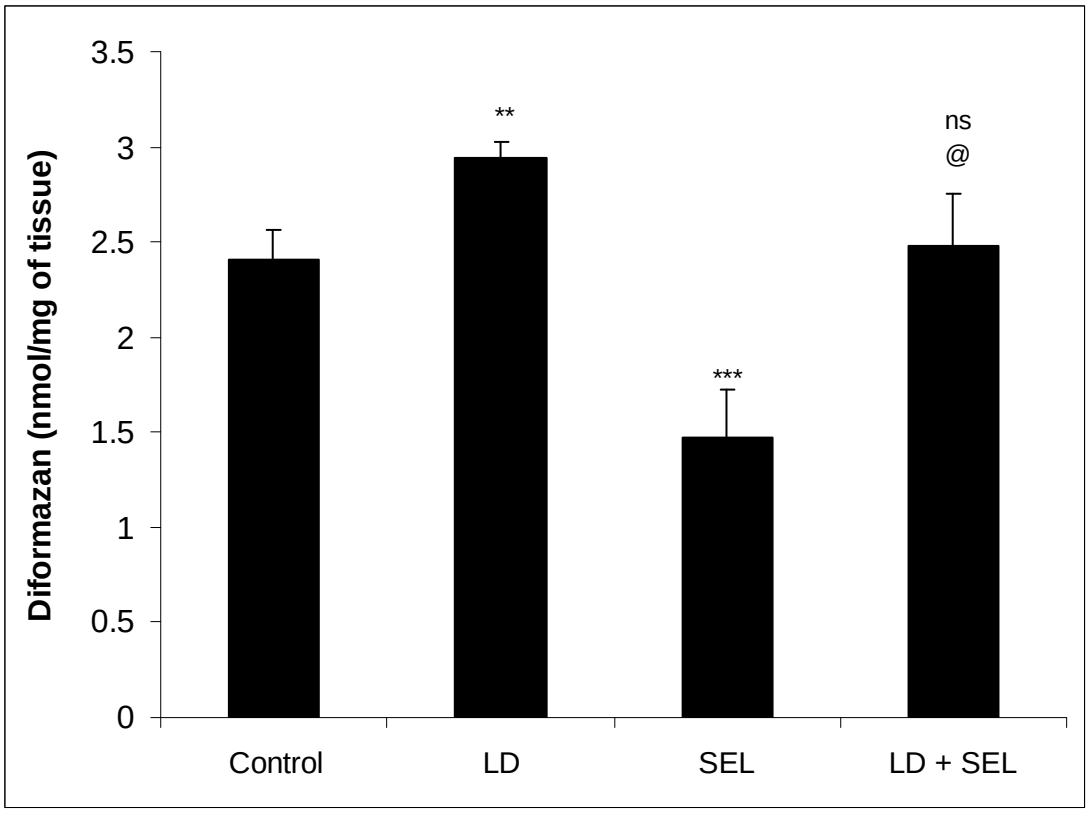


Figure 5.8: The effect of acute LD and SEL treatment on endogenous $O_2^{\bullet-}$ formation. Each bar represents the mean ± SD (n = 5). ns ($p > 0.05$), ** ($p < 0.01$) and *** ($p < 0.001$) compared to the control values; @ ($p < 0.01$) compared to the LD treatment group.

5.4.4 DISCUSSION

The results of this study show that the systemic administration of LD results in a significant increase in $O_2^{\bullet-}$. Whether this increase is caused by LD itself or because it is metabolized to DA, which may in turn lead to the formation of 6-OHDA is unclear. All three of these compounds (LD, DA and 6-OHDA) are known to generate a number of different free radicals, including $O_2^{\bullet-}$ during their auto-oxidation. In addition to this these agents have all been shown to inhibit specific components of the mitochondrial respiratory chain, the main source of $O_2^{\bullet-}$ production *in vivo*.

Superoxide dismutase (SOD) provides the first line of defense against oxidative stress by catalyzing the dismutation of $O_2^{\bullet-}$ into H_2O_2 and O_2. H_2O_2 is also formed during the MAO catalyzed metabolism of DA (figure 5.9). H_2O_2 is normally a harmless cellular metabolite that is converted to water by catalase and/or selenium-dependent glutathione peroxidase (Jain *et al*., 1991). In the brain glutathione peroxidase is considerably more important than catalase because of the low activity of the latter enzyme in most parts of the CNS (Jain *et al*., 1991). In the presence of Fe^{2+}, however, H_2O_2 can be converted via the Fenton reaction to •OH, a much more reactive and tissue damaging species than $O_2^{\bullet-}$. Since the systemic administration of LD increases $O_2^{\bullet-}$ formation in the striatum it could theoretically also result in an increase in striatal hydroxyl radical formation. This will be investigated in the following chapter.

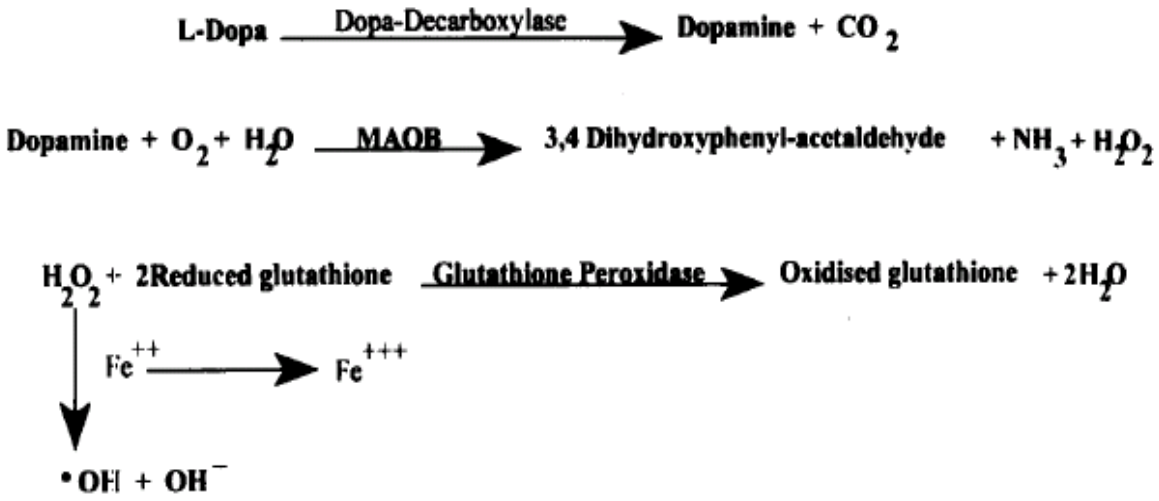


Figure 5.9: The generation of H_2O_2 and •OH after the administration of LD (Ebadi *et al*., 1995)

The results of the present study show that SEL treatment is able to suppress striatal $O_2^{\bullet-}$ production below that of control values. In addition to this, the combined treatment of rats with LD and SEL results in significantly lower $O_2^{\bullet-}$ production compared to that observed in rats receiving only LD. Some of the possible reasons for this reduced $O_2^{\bullet-}$ production by SEL have been enumerated in the previous experiment, however another possible reason is that SEL may be involved in the up-regulation of $O_2^{\bullet-}$ metabolizing systems. For example, Knoll (1988) reported that the subchronic administration of SEL

(2 mg/kg) i.p. for three weeks resulted in a ten-fold increase in soluble SOD activity, exclusively in the striatum. Other researchers have reported smaller increases in soluble SOD activity induced by SEL (Carrillo *et al*., 1991; Clow *et al*., 1991). However, an increase in the activity of SOD would result in an increase in the conversion of $O_2^{\bullet-}$ to H_2O_2. The produced H_2O_2 could then result in an increase in •OH production by participation in the Fenton reaction. An increase in SOD could therefore indirectly result in an increase in •OH formation. Increased SOD activity would therefore not be expected to offer neuroprotection unless the activities of catalase or glutathione peroxidase, which convert hydrogen peroxide to water, were also increased. Studies have shown that SEL has no effect on the activity of glutathione peroxidase but elevates catalase activity (Carrillo *et al*., 1991; Pattichis *et al*., 1995). However, the elevation of catalase activity was shown to occur at doses somewhat higher than those needed for MAO-B inhibition. The following chapter will therefore investigate whether the doses of SEL used in the present study elevate or reduce the striatal production of •OH.

CHAPTER SIX

HYDROXYL RADICAL FORMATION

6.1 INTRODUCTION

Cytotoxic polyphenols and reactive oxygen species, such as superoxide anion radicals ($O_2^{\bullet -}$) and hydroxyl radicals (•OH), formed within catecholamine neurons during the auto-oxidation of DA and other brain catecholamines has been implicated as causal or contributing factors in Parkinson's disease (Obata, 2002). A major concept concerning (per)oxidations of lipids, proteins, nucleic acids and other important biomolecules mediated by ROS, centers on the activity of the •OH radical, the most reactive oxygen species generated via the iron-catalyzed Fenton reaction (Floyd and Lewis, 1983, Aust *et al*., 1993) or alternatively via nitric oxide related mechanisms (Hammer *et al*., 1993). Neuroprotective mechanisms against catecholamine-derived ROS include the enzymatic degradation of H_2O_2 by catalase and glutathione peroxidase, scavenging of ROS by antioxidant molecules such as ascorbate and reduced glutathione, alternative metabolic pathways that reduce or prevent the ability of catecholamines to undergo auto-oxidation and redox cycling and the synthesis of neuromelanin which may sequester reactive metal ions, trap ROS and act as a redox buffer against both reducing and oxidizing molecules (Nappi and Vass, 1998)

Electron spin resonance (ESR) trapping (Pou *et al*., 1989) and aromatic hydroxylation assays (Halliwell *et al*., 1989) provide the most specific and direct measurements of reactive oxygen species (ROS). Using biologically relevant *in vitro* •OH generating systems it was demonstrated (Richmond *et al*., 1981) that salicylate could trap •OH to form 2,3-dihydroxybenzoic acid (2,3-DHBA, ± 49 %), 2,5-dihydroxybenzoic acid (2,5-DHBA, ± 40 %) as well as catechol (± 11 %) (Grootveld and Halliwell, 1986). Since these studies, •OH formation has also been investigated *in vivo* by using either intracerebral or systemic administration of salicylic acid as a •OH trapping agent.

The scavenging of •OH by DA results in the formation of 6-OHDA, 5-OHDA and 2-OHDA. The scavenging of •OH by DA may protect biomolecules from the damaging effects of this radical. However, both DA and 6-OHDA have the potential to enhance •OH formation during their auto-oxidation and metabolism. This chapter investigates the net effect of DA on •OH generation by the Fenton system using a rapid and sensitive HPLC-ECD method for the simultaneous detection of 2,3-DHBA, 2,5-DHBA and catechol (CAT). Chapter 4 showed that intraperitoneal administration of LD results in a significant elevation of striatal DA levels. The effect of DA on •OH generation by the Fenton system *in vitro* was therefore compared to the effect that LD administration had on •OH production *in vivo*. In each study SEL was used to examine its ability to trap •OH. SEL contains a phenyl ring and studies indicate that it should be able to trap •OH but that its ability to provide protection depends upon the biological target (Thomas *et al*., 1997).

6.2 EFFECT OF DOPAMINE ON HYDROXYL RADICAL GENERATION VIA IRON MEDIATED FENTON CHEMISTRY *IN VITRO*

6.2.1 INTRODUCTION

Chapter 2 demonstrates that DA is able to scavenge •OH produced via the Fenton reaction to form 6-OHDA. Scavenging of •OH by DA also results in the formation of 2-OHDA and 5-OHDA which are not cytotoxic (Slivka and Cohen, 1985). However, DA and 6-OHDA also have the capacity to generate this free radical during their auto-oxidation and metabolism. Therefore, in an attempt to assess the potential of DA to cause oxidative stress, this study investigates whether DA inhibits or enhances hydroxyl radical generation under Fenton conditions. This study also investigates the effect of ascorbic acid on the capacity of DA to generate •OH under Fenton conditions.

6.2.2 MATERIALS AND METHODS

6.2.2.1 Chemicals and reagents

DA hydrochloride, sodium salicylate, 2,3-dihydroxybenzoic acid (2,3-DHBA), 2,5-dihydroxybenzoic acid (2,5-DHBA) and catechol (CAT) were purchased from the Sigma Chemical Corporation, St. Louis, MO, USA. EDTA and ferrous sulfate were purchased from Merck, Darmstadt, Germany. All other chemicals were of the highest quality available from commercial sources.

6.2.2.2 Hydroxyl radical generating system

The Fe(II)-EDTA/H_2O_2 system was used as the •OH generating system. The •OH generating components were prepared by using the following sequence of additions and final concentrations of reagents: water, phosphate buffer (final concentration 50 mM, pH 7.4), hydrogen peroxide (100 µM), EDTA (240 µM) and ferrous sulfate (200 µM). Incubations with varying concentrations of DA were performed at 22 ºC for a period of two hours. At various time periods (20, 40, 60, 90 and 120 minutes), aliquots (0.1 ml) of reaction mixture were removed and quenched in 0.9 ml of ice cold 0.1 M $HClO_4$. The effect of ascorbic acid concentration on •OH formation by DA was evaluated by adding various concentrations (0.01, 0.1, 1 mM) of ascorbate before the addition of DA. In reaction mixtures containing 0.1 mM and 1 mM of ascorbate aliquots of 0.01 ml rather than 0.1 ml were removed from the reaction mixture and quenched in 0.99 ml of ice cold 0.1 M $HClO_4$. The samples were analysed for the three formed salicylate hydroxylation products 2,3-DHBA, 2,5-DHBA and CAT using high performance liquid chromatography with electrochemical detection. Final results are expressed as nmol/ml.

6.2.2.3 Instrumentation

As described in section 2.2.2.3

6.2.2.4 Chromatographic conditions

Separation was achieved using an Ultrasphere C18 IP 80A analytical column (5 µm, 250 x 4.6 mm). Each liter of mobile phase contained 1.5 g heptane sulfonic acid, 0.1 g of EDTA, 3 ml of triethylamine and 125 ml of acetonitrile dissolved in Milli-Q water. The pH of the solution was adjusted to 2.8 with 3 ml of phosphoric acid before the addition of the acetonitrile (Obata and Yamanaka, 1996). The mobile phase was degassed

twice using a 0.45 µm membrane filter prior to use. The flow rate was 0.8 ml/min, and the electrodetection was performed at 0.65 V. Results are expressed as nmol/ml

6.2.2.5 Statistical analysis

The differences in the means were analysed using a one way analysis of variance (ANOVA) followed by the Student Newman-Keuls Multiple Range Test. The level of significance was set at $p < 0.05$.

6.2.3 RESULTS

Hydroxyl radical generation by the Fe(II)-EDTA/H_2O_2 system (Fenton reaction) employed throughout this investigation was linear with time for at least 2 hours. Figure 6.1 is a series of chromatograms showing that in the presence of DA, •OH generation was found to be enhanced in a concentration-dependent manner. In incubations containing as little as 20 nmol (0.002 mM final concentration) of DA, •OH production was approximately double that observed in control reactions lacking DA. In incubations containing 50 nmol (0.005 mM final concentration) of DA, •OH formation was nearly three times higher than the control values.

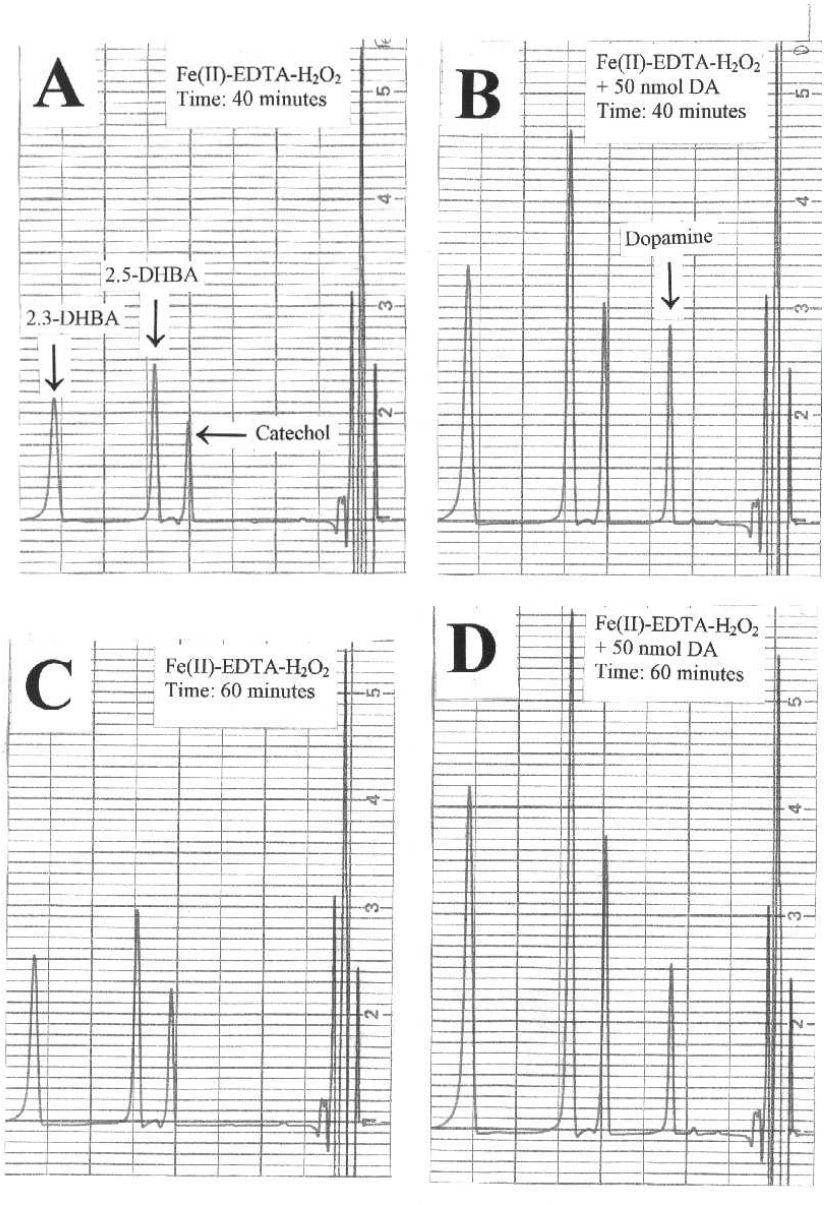


Figure 6.1: Representative chromatograms showing the concentration dependent enhancing effect of DA on the production of •OH by Fenton chemistry (0.1 ml aliquots of the reaction mixture where quenched in 0.9 ml of 0.1 M $HClO_4$.) The products of salicylate hydroxylation (2,3-DHBA, 2,5-DHBA and CAT) are indicators of •OH production.

Figure 6.2 shows the formation of the hydroxylation products of salicylate (2,3-DHBA, 2,5-DHBA and CAT) with time in the Fe(II)-EDTA/H_2O_2 system.

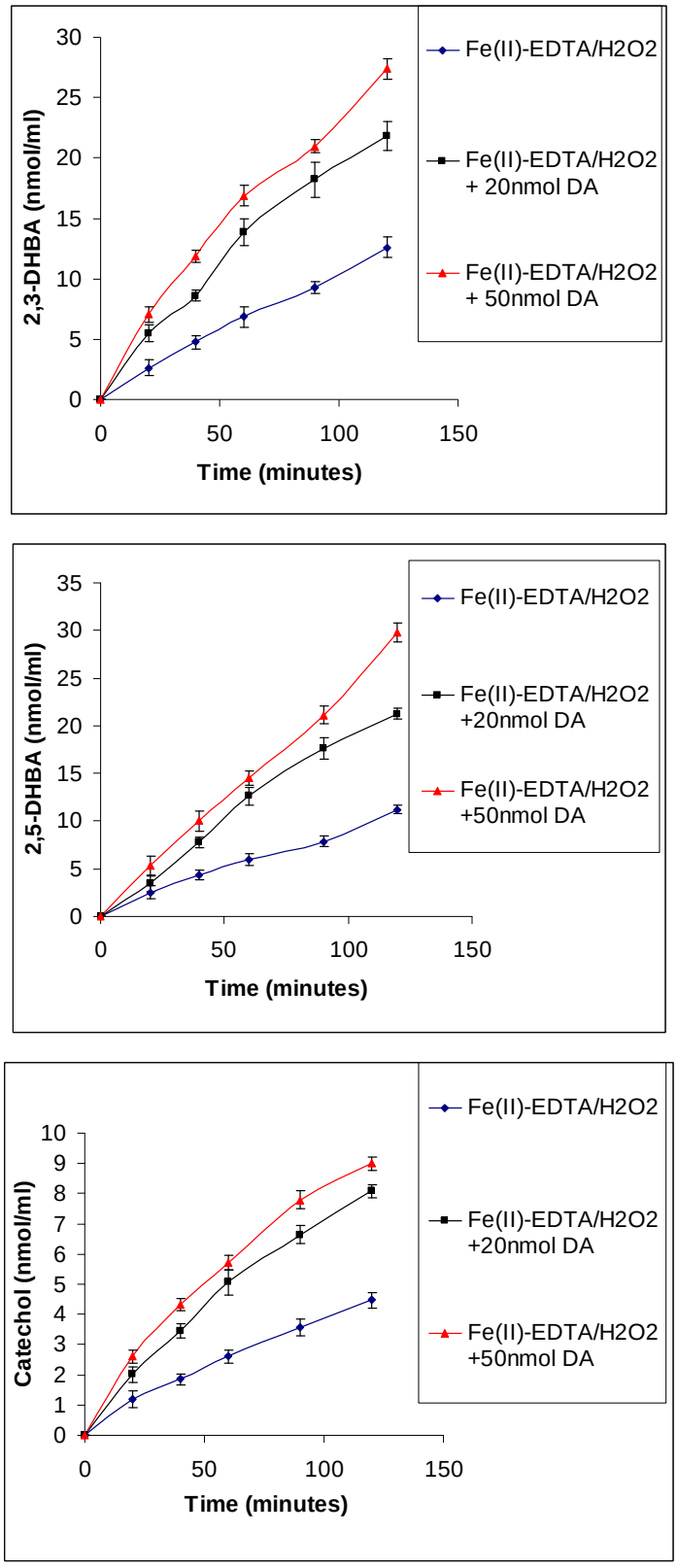


Figure 6.2: The formation of 2,3-DHBA, 2,5-DHBA and CAT with time in the Fe(II)-EDTA/H_2O_2 system in the absence and presence of DA (20 nmol and 50 nmol).

The addition of ascorbic acid to the reaction mixture resulted in a pronounced increase in hydroxyl radical formation, however the ascorbic acid reduced the rise in •OH production induced by DA and at a concentration of 1 mM, the ascorbic acid prevented the DA induced rise in •OH production completely (figure 6.3, 6.4, 6.5 and 6.6).

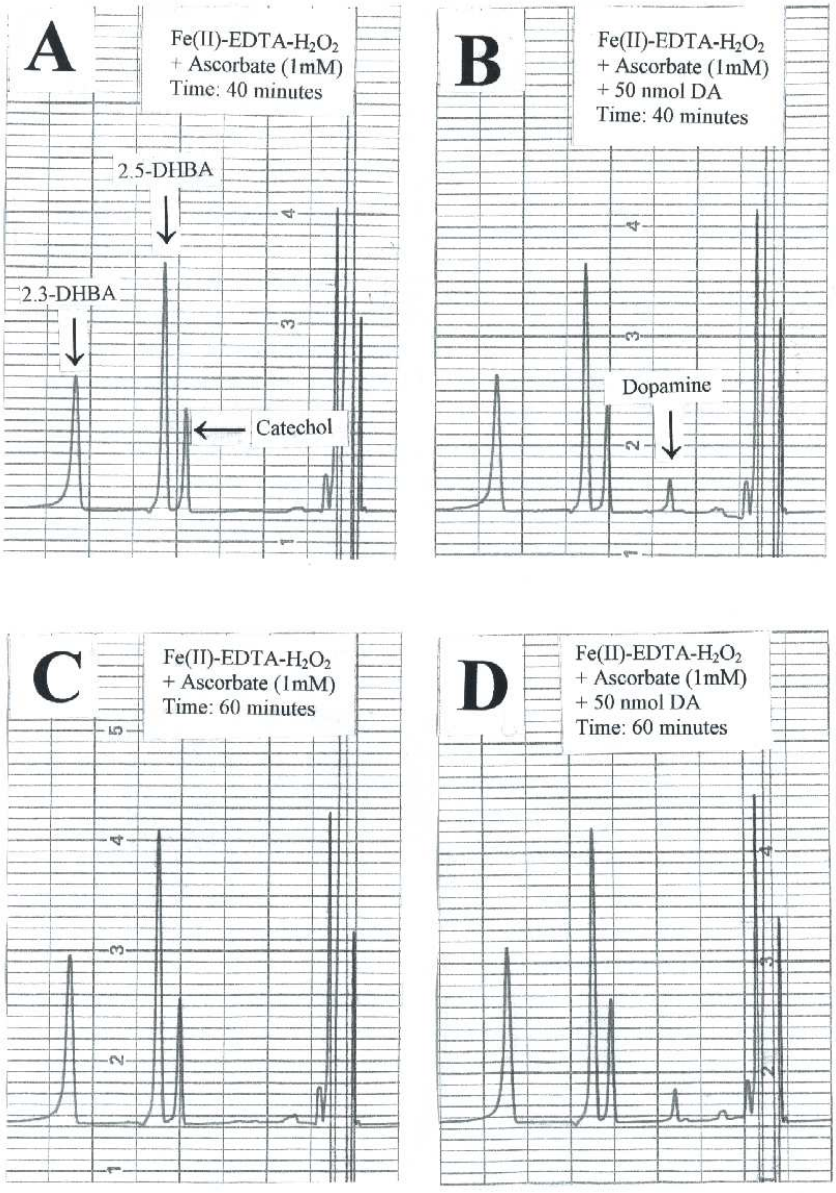


Figure 6.3: Representative chromatograms showing the effect of ascorbate on the DA enhanced production of •OH by Fenton chemistry (0.01 ml aliquots of the reaction mixture where quenched in 0.99 ml of 0.1 M $HClO_4$.)

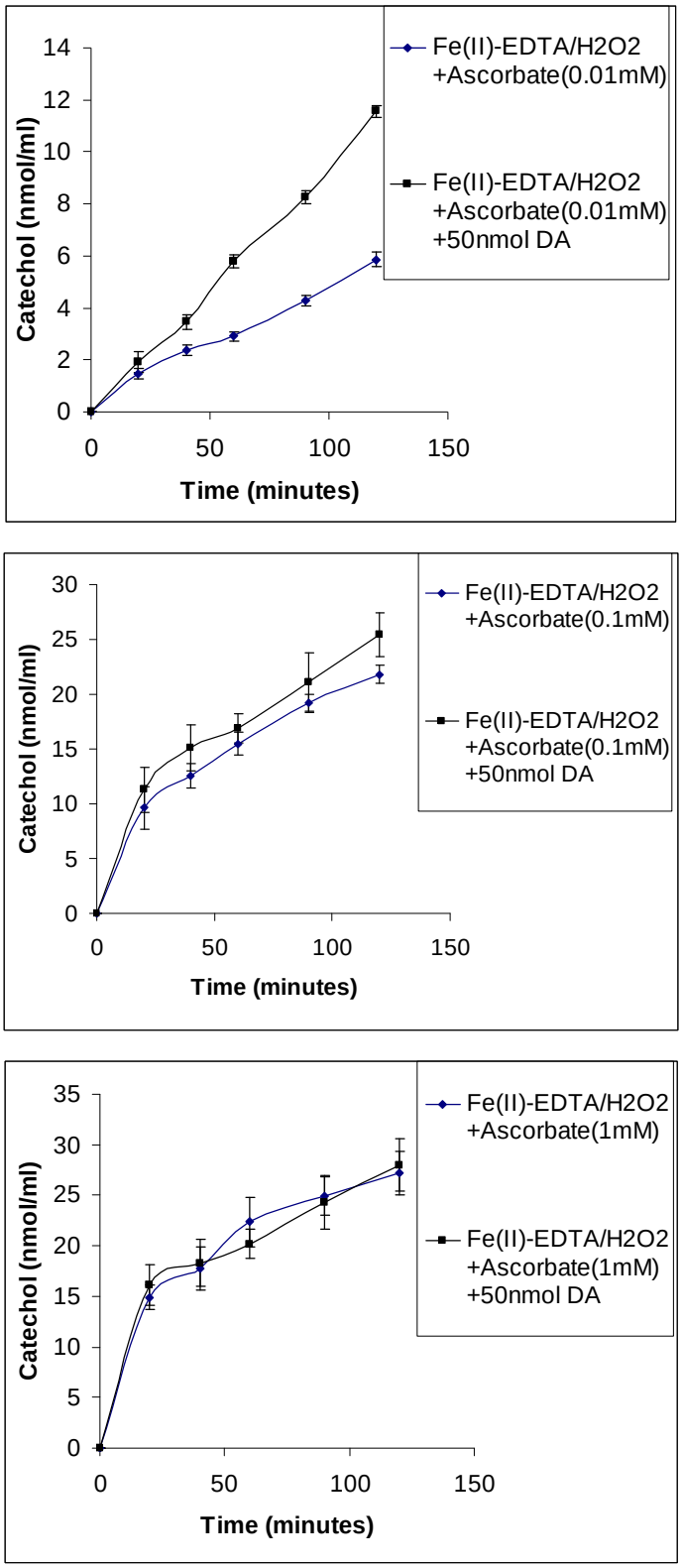


Figure 6.4: The formation of CAT with time in the Fe(II)-EDTA/H_2O_2 system after the addition of ascorbate (0.01 mM, 0.1 mM and 1 mM).

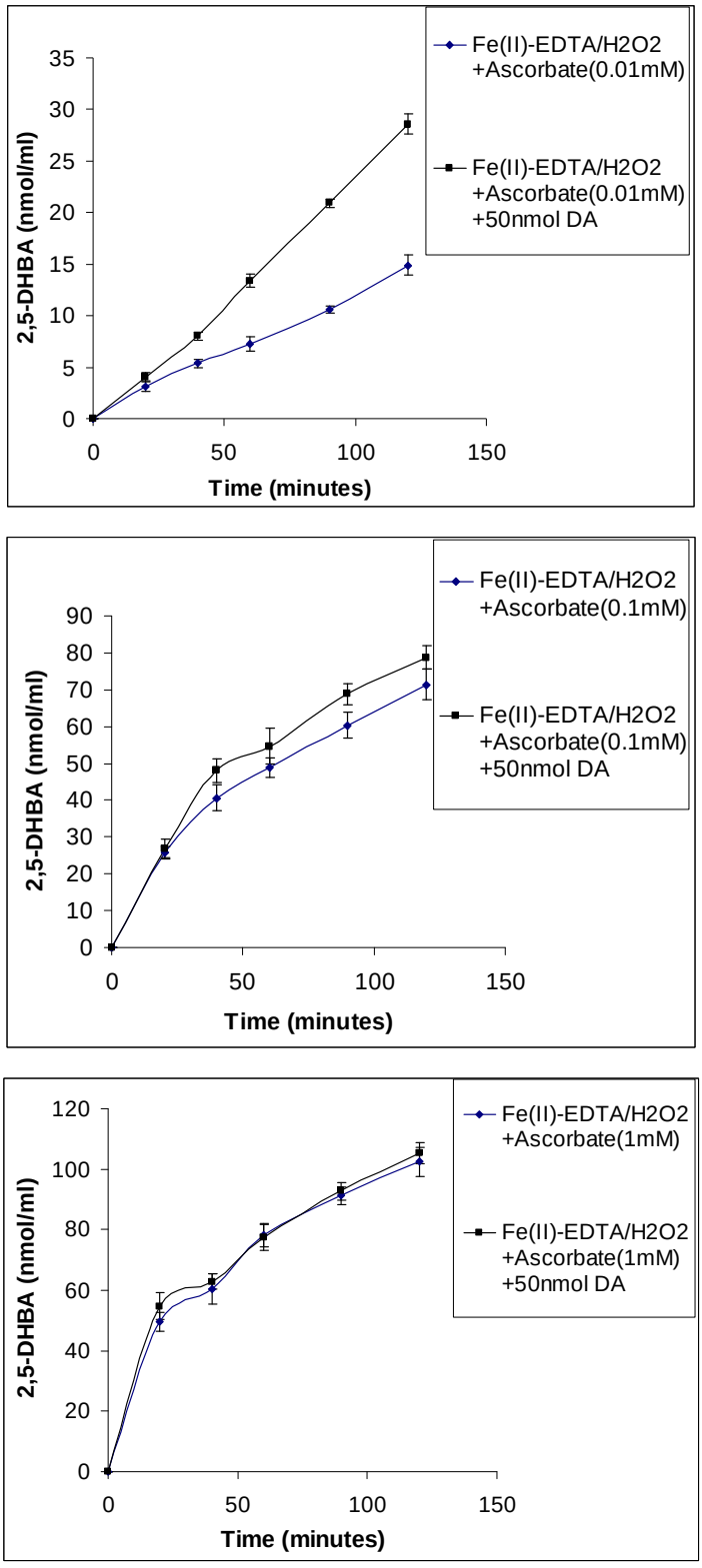
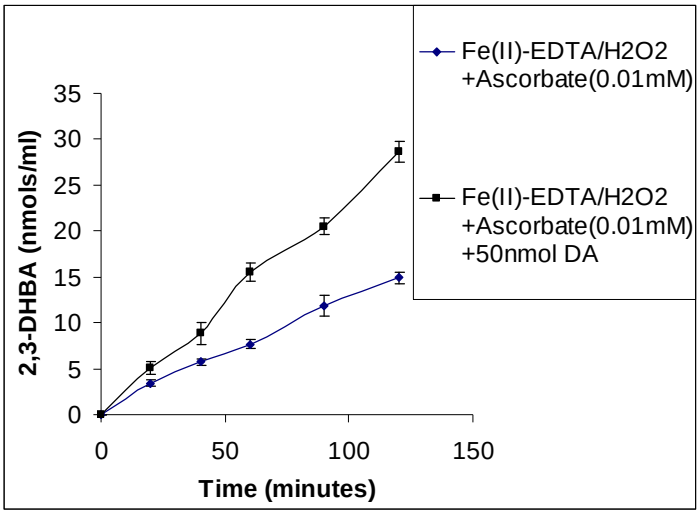
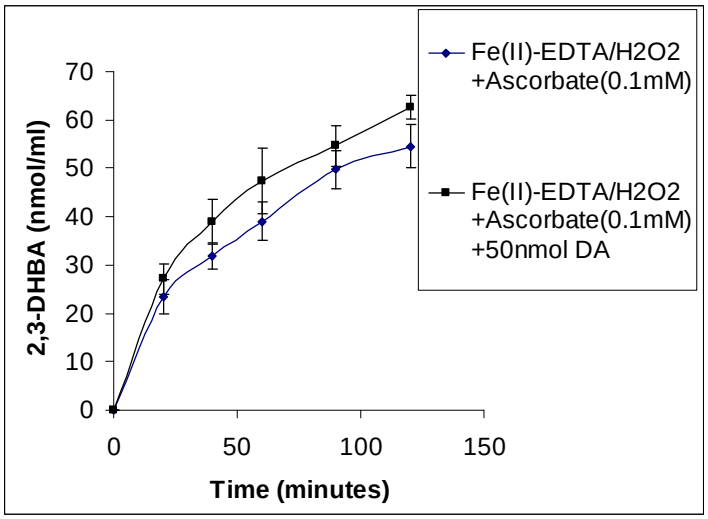


Figure 6.5: The formation of 2,5-DHBA with time in the Fe(II)-EDTA/H_2O_2 system after the addition of ascorbate (0.01 mM, 0.1 mM and 1 mM).





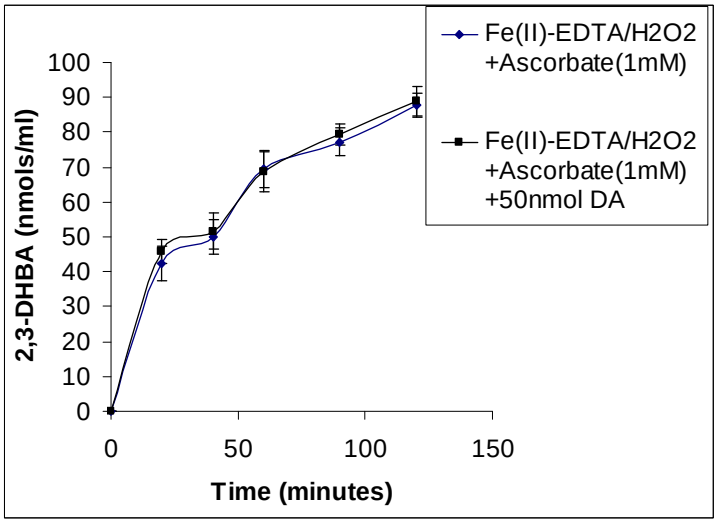


Figure 6.6: The formation of 2,3-DHBA with time in the Fe(II)-EDTA/H_2O_2 system after the addition of ascorbate (0.01 mM, 0.1 mM and 1 mM).

6.2.4 DISCUSSION

The results of this investigation establish that DA significantly enhances the production of •OH by the Fenton system *in vitro*. It is interesting to note that similar results were previously obtained with 6-OHDA (Méndez-Álverez *et al*., 2001). Although chapter 2 demonstrates that DA has the potential to trap •OH, the present report of the ability of DA to generate this free radical under the same conditions suggests that the scavenging of •OH by DA to form 2-OHDA, 5-OHDA and 6-OHDA is unlikely to have any neuroprotective effects under Fenton conditions.

The enhanced •OH production may be a consequence of the auto-oxidation of DA, as well as any 6-OHDA formed during the course of the experiment. The generally accepted mechanism for the production of •OH during the auto-oxidation of DA is the following (Li *et al*., 1995):

$$\text{Catechol} + O_2 \rightarrow SQ\bullet + O_2^{\bullet-} \qquad \text{(Equation 6.1)}$$

$$SQ\bullet + O_2 \rightarrow \text{quinone} + O_2^{\bullet-} \qquad \text{(Equation 6.2)}$$

$$\text{Catechol} + O_2^{\bullet-} + H^+ \rightarrow SQ\bullet + H_2O_2 \qquad \text{(Equation 6.3)}$$

Catechol containing compounds, such as DA and 6-OHDA are oxidized by oxygen (equation 6.1) to yield an intermediary semiquinone (SQ•) and eventually the corresponding ortho-quinone (equation 6.2). Superoxide radical anions ($O_2^{\bullet-}$) are formed during both these reactions. These $O_2^{\bullet-}$ also oxidize DA and 6-OHDA, in which event H_2O_2 is formed (equation 6.3). H_2O_2 participates in the Fenton reaction (equation 6.4) to finally give •OH.

The substantial increase in •OH generation after the addition of DA to the Fenton reaction is also probably a consequence of the ability of iron to catalyze the above mentioned auto-oxidation of DA according to the following mechanism (Linert and Jameson, 2000):

$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^- + \bullet OH$ (Equation 6.4)

$Fe^{3+} + Catechol \rightarrow Fe^{2+} + SQ\bullet$ (Equation 6.5)

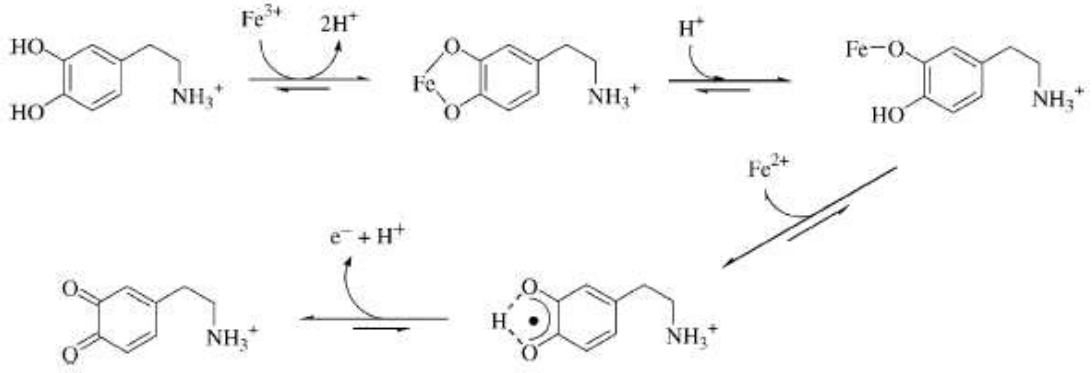


Figure 6.7: Reaction mechanism by which Fe^{3+} catalyses the auto-oxidation of DA (Hermida-Ameijeiras *et al*., 2004).

The Fe^{2+} formed in this way could then also be free to participate in the Fenton reaction. Iron binding studies will be done in a later chapter to investigate whether this Fe^{2+} is free to participate in the Fenton reaction or whether it is bound by DA. Any 6-OHDA formed during the course of the experiment would also undergo iron-catalyzed auto-oxidation in the same way, however the rate of auto-oxidation of 6-OHDA is likely to be even faster than that for DA.

The above hypothesis is supported by the fact that the DA induced rise in •OH production by the Fenton system is inhibited in a concentration dependent manner by the addition of ascorbic acid to the system. The addition of ascorbic acid results in a profound increase in the overall production of •OH by the Fenton system, this is best understood by the ability of ascorbate to promote the recycling of iron from its ferric to ferrous states, however the ascorbic acid prevents the rise in •OH caused by DA. This effect is probably due to the fact that ascorbic acid inhibits the auto-oxidation of DA and 6-OHDA and therefore prevents the rise in •OH formation associated with the auto-oxidation of these compounds, when present at high enough concentrations.

Chapter 4 showed that LD administration results in a significant rise in striatal DA content, this together with relatively high levels of iron and H_2O_2 in the striatum may result in a significant rise in •OH production. This investigation also shows that any detectable rise in •OH levels induced by exogenous LD administration is highly dependent on local tissue ascorbate levels. The effect of LD administration on striatal •OH production *in vivo* will be investigated in a following experiment.

6.3 EFFECT OF SELEGILINE ON HYDROXYL RADICAL FORMATION VIA IRON MEDIATED FENTON CHEMISTRY *IN VITRO*

6.3.1 INTRODUCTION

PD is typified by a significant loss of DA in the nigrostriatal pathway. In an effort to maintain brain DA levels, prevention of the catabolism of DA by MAO-B led to the development of SEL, a selective irreversible inhibitor of the enzyme (Thomas *et al*., 1997). However, evidence has also been presented that SEL may protect the brain from oxidative insult by virtue of its antioxidant activity (Chiueh *et al*., 1994 and Thomas *et al*., 1997). Chapter 2 showed that SEL was able to suppress the formation of 6-OHDA *in vitro.* This effect may be a consequence of its •OH scavenging properties. This study investigates the •OH trapping ability of SEL by investigating the effect of SEL on •OH formation by Fenton chemistry as well as examining the effect of SEL on the DA enhanced generation of •OH by this system observed in the previous study.

6.3.2 MATERIALS AND METHODS

6.3.2.1 Chemicals and reagents

SEL hydrochloride, DA hydrochloride, sodium salicylate, 2,3-dihydroxybenzoic acid (2,3-DHBA), 2,5-dihydroxybenzoic acid (2,5-DHBA) and CAT were purchased from the Sigma Chemical Corporation, St. Louis, MO, USA. EDTA and ferrous sulfate were purchased from Merck, Darmstadt, Germany. All other chemicals were of the highest quality available from commercial sources.

6.3.2.2 Hydroxyl radical generating system

The same Fe(II)-EDTA/H_2O_2 system used in the previous experiment was used as the •OH generating system. The •OH generating components were prepared as described in section 5.2.2.2. Incubations with varying concentrations of SEL were performed at 22ºC for a period of two hours. At various time periods (20, 40, 60, 90 and 120 minutes), aliquots (0.1 ml) of reaction mixture were removed and quenched in 0.9 ml of ice cold 0.1 M $HClO_4$. The effect of SEL on enhanced •OH formation induced by DA was evaluated by adding various concentrations (0.1 mM or 1 mM) of SEL before the addition of DA. The samples were analysed for the three formed salicylate hydroxylation products 2,3-DHBA, 2,5-DHBA and CAT using high performance liquid chromatography with electrochemical detection. Final results are expressed as nmol/ml.

6.3.2.3 Instrumentation

As described in section 2.2.2.3

6.3.2.4 Chromatographic conditions

As described in section 6.2.2.4

6.3.2.5 Statistical analysis

As described in section 6.2.2.5

6.3.3 RESULTS

Figure 6.8 is a series of chromatograms showing that when 10 µmol of SEL was added to the reaction mixtures containing Fe(II)-EDTA/H_2O_2, •OH production is diminished significantly relative to control incubations without SEL ($p < 0.001$).

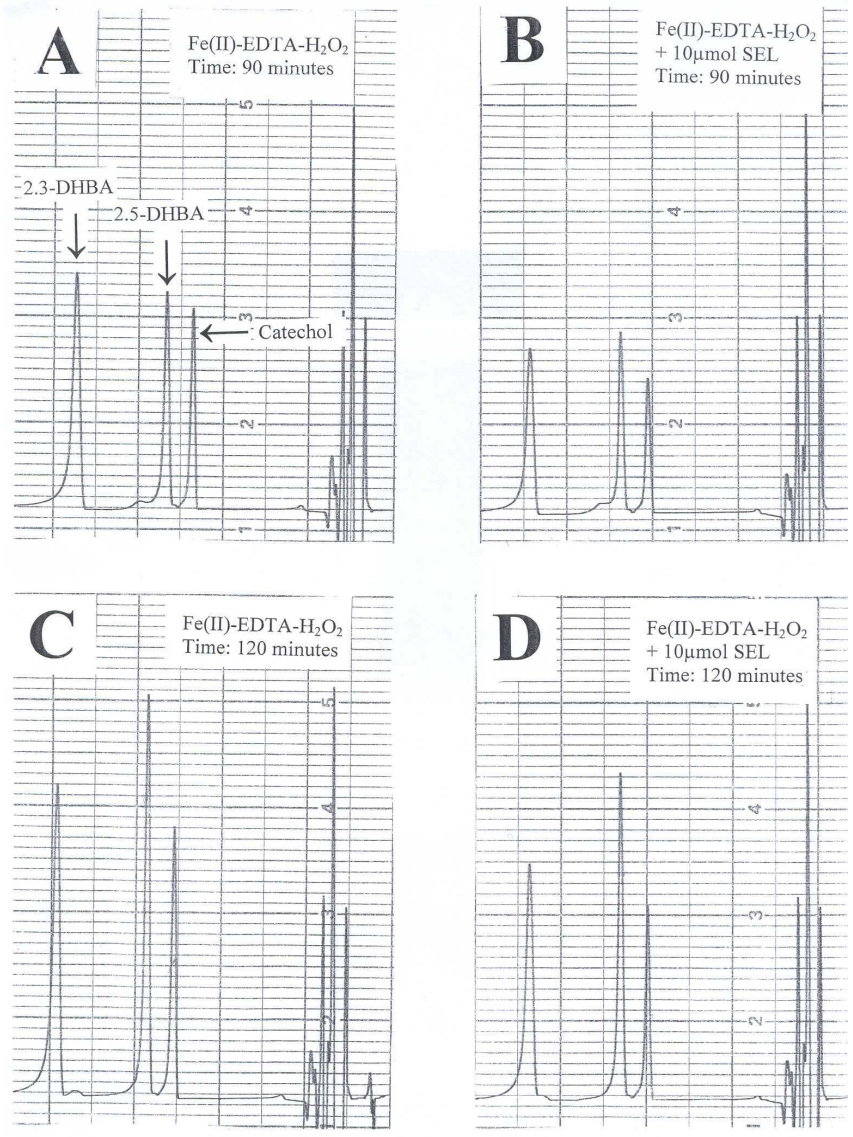


Figure 6.8: Representative chromatograms showing the effect of SEL on the production of •OH by Fenton chemistry (0.1 ml aliquots of the reaction mixture where quenched in 0.9 ml of 0.1 M $HClO_4$.)

By taking into account the formation of 2,3-DHBA, 2,5-DHBA and CAT, results show •OH production by Fe(II)-EDTA/H_2O_2 to be reduced by approximately 22 % in mixtures containing 10 µmol of SEL (final concentration: 1 mM). Reaction mixtures containing 1 µmol of SEL (final concentration: 0.1 mM) showed no significant reduction in •OH production relative to control incubations. Figure 6.9 shows the formation of the hydroxylation products of salicylate (2,3-DHBA, 2,5-DHBA and CAT) with time in the Fe(II)-EDTA/H_2O_2 system in the presence and absence of different concentrations of SEL.

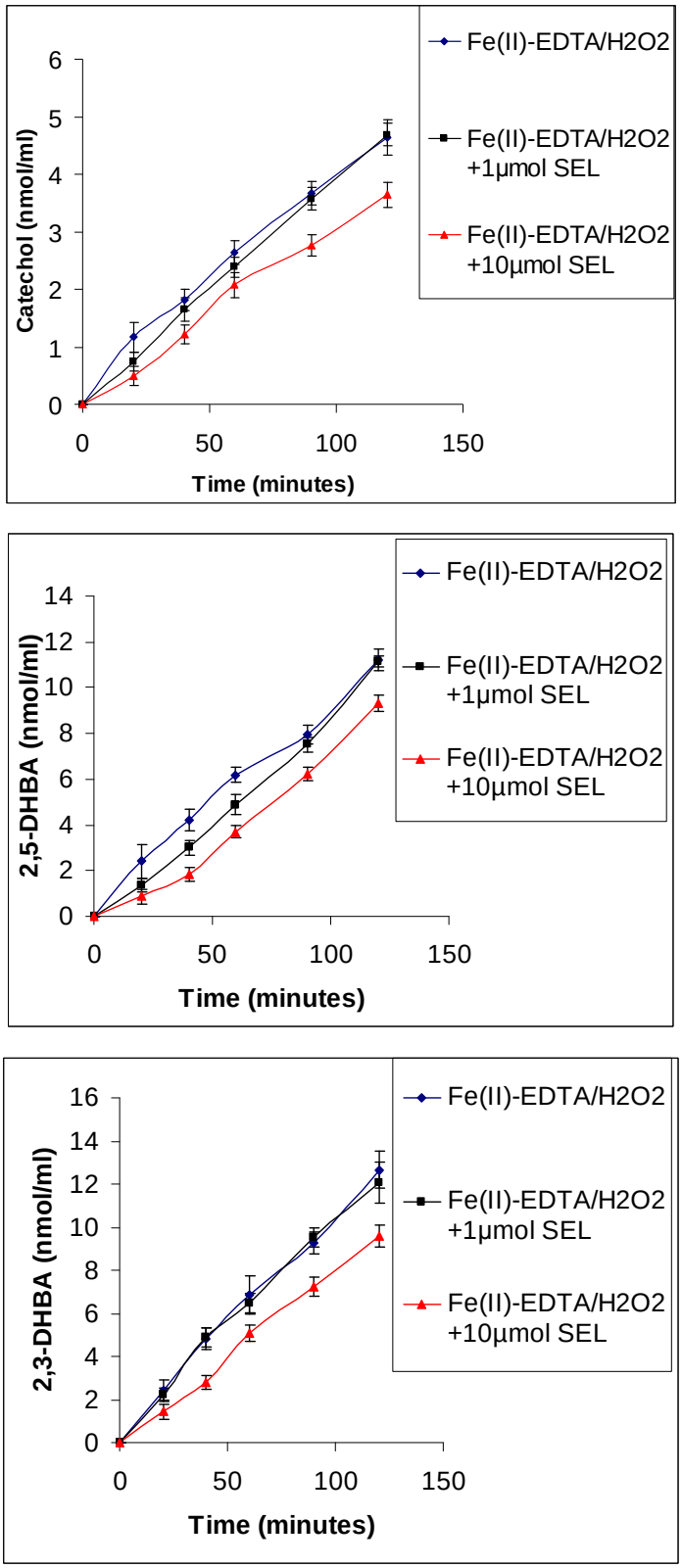


Figure 6.9: The formation of 2,3-DHBA, 2,5-DHBA and CAT with time in the Fe(II)-EDTA/H_2O_2 system in the absence and presence of SEL (1 µmol and 10 µmol)

Figure 6.10 is a series of chromatograms showing that the addition of 10 µmol of SEL to reactions mixtures containing 50 nmol of DA together with the Fe(II)-EDTA/H_2O_2 complex results in a significant decrease in •OH production relative to incubations without SEL ($p < 0.001$).

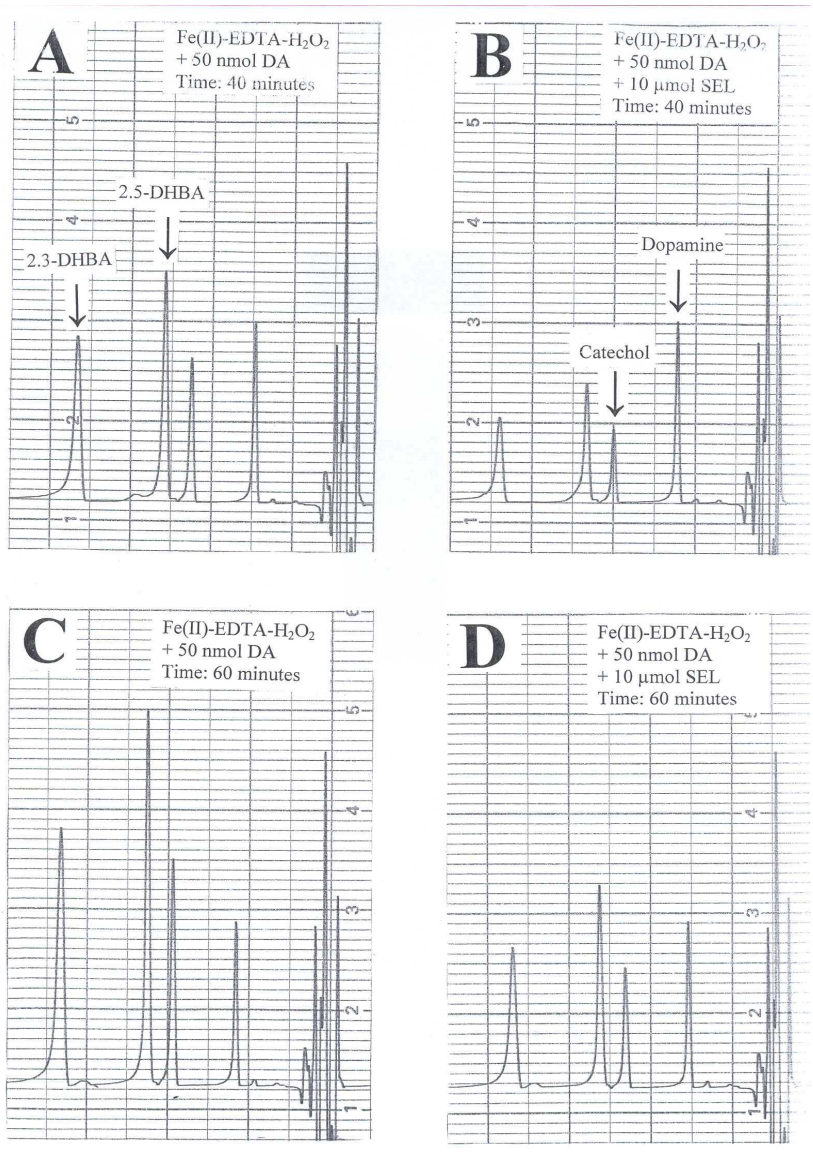


Figure 6.10: Representative chromatograms showing the effect of SEL on enhanced production of •OH induced by DA (0.1 ml aliquots of the reaction mixture where quenched in 0.9 ml of 0.1 M $HClO_4$.)

Analysis of 2,3-DHBA, 2,5-DHBA and CAT formation over time showed that the enhanced production of •OH induced by the addition of 50 nmol of DA to the Fe(II)-EDTA/H_2O_2 reaction system is reduced by approximately 40 % in incubation mixtures containing 10 µmol of SEL (figure 6.11). The addition of 1 µmol of SEL (final concentration: 0.1 mM) to the same system resulted in no significant change in •OH production.

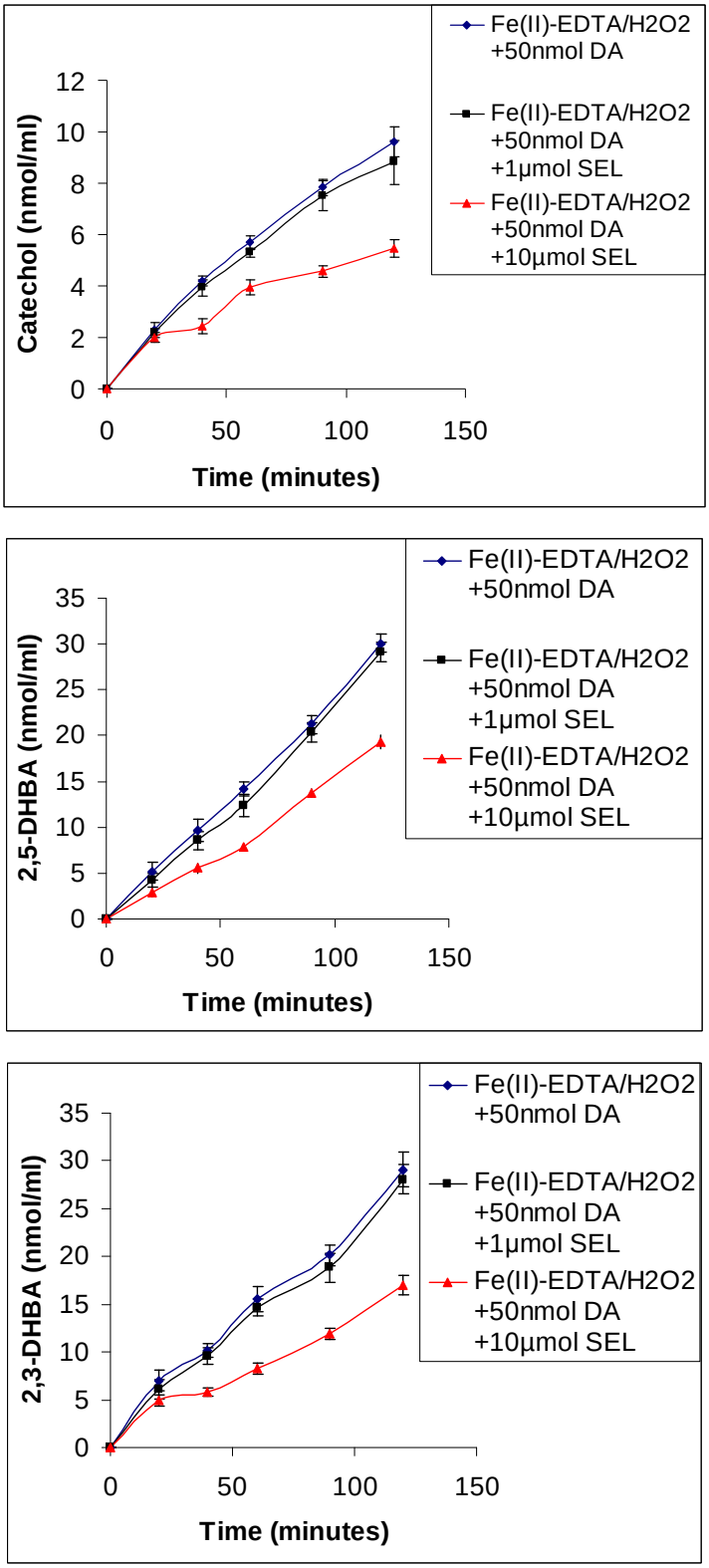


Figure 6.11: The effect of SEL on the increased formation of 2,3-DHBA, 2,5-DHBA and CAT induced by the addition of DA to Fe(II)-EDTA/H_2O_2.

6.3.4 DISCUSSION

The results of this investigation demonstrate that SEL can trap •OH. The results also show a greater inhibition of •OH formation (approximately 40 %) by SEL in Fenton reaction mixtures containing DA than those lacking DA (approximately 20 % inhibition). This observation suggests that SEL may interfere with the iron catalyzed auto-oxidation of DA. The iron binding properties of SEL will be investigated in a later chapter.

The trapping of •OH may account for the decrease in 6-OHDA formation observed after the addition of SEL to reaction mixtures in chapter 2. However, it should be mentioned that relatively high concentrations of SEL (1 mM or more) are needed to inhibit •OH formation by Fenton chemistry and that it therefore appears that SEL is only moderately reactive with •OH. Given the extreme reactivity of •OH it is not surprising that SEL can trap this radical. Whether or not the antioxidant activity of SEL has biological significance will be addressed by evaluating the effect of SEL on •OH induced damage to lipids, proteins and antioxidant enzymes in chapters seven, eight and nine.

In addition to its direct free radical scavenging activity, SEL exerts a number of other neuroprotective actions *in vivo*. Inhibition of MAO-B diminishes the formation of H_2O_2 formed during the metabolism of DA. In addition to this, SEL has the ability to up-regulate antioxidant enzymes, such as catalase and superoxide dismutase (Carrillo *et al*., 1991, 1992; Thomas *et al*., 1997). The effect of SEL on •OH formation in the striatum of male Wistar rats, both on its own and in combination with LD treatment will be investigated in the following experiment.

6.4 EFFECT OF L-DOPA AND SELEGILINE TREATMENT ON STRIATAL HYDROXYL RADICAL FORMATION *IN VIVO*

6.4.1 INTRODUCTION

LD is the primary, most effective and most widely used drug in the treatment of PD (LeWitt, 1989). However, the debate about the toxicity of LD to dopaminergic neurons has not yet been resolved and has led some clinicians to delay the commencement of LD therapy (Fahn, 1996; Jenner and Brin, 1998). Several *in vitro* studies have shown that LD is toxic to dopaminergic neurons in culture (Mena *et al*., 1992, 1993; Mytilineou *et al*., 1993; Pardo *et al*., 1995a). This toxicity has been attributed to the production of hydrogen peroxide and oxygen free radicals generated through the metabolism and auto-oxidation of LD and DA (Graham *et al*., 1978, Han *et al*., 1996). Even though numerous *in vitro* studies have reported LD induced oxidative stress and toxicity to dopaminergic neurons, few studies have investigated the generation of oxygen free radicals following LD administration *in vivo* (Fahn, 1996). This study investigates whether acute LD administration results in enhanced hydroxyl radical generation in the striatum of male Wistar rats. The study also investigates the effect of SEL on striatal •OH formation. We used the technique of *in vivo* salicylate trapping for detecting •OH formation. The reaction of •OH with salicylate results in the formation of 2,3-DHBA and 2,5-DHBA. 2,5-DHBA can also be generated by the cytochrome P450 system (Halliwell *et al*., 1991) and 2,3-DHBA is therefore considered to be a more specific marker of •OH generation *in vivo* than 2,5-DHBA.

6.4.2 MATERIALS AND METHODS

6.4.2.1 Chemicals and reagents

L-3,4-dihydroxyphenylalanine (LD), benserazide hydrochloride, SEL hydrochloride, sodium salicylate, 2,3-DHBA, 2,5-DHBA, CAT, norepinepherine (NE) homovanillic acid (HVA), 3,4-dihydroxyphenylacetic acid (DOPAC), 5-hydroxytryptamine creatinine sulfate (5-HT) and 5-hydroxyindole-3-acetic acid (5-HIAA) were purchased from the Sigma Chemical Corporation, St. Louis, MO, USA. All other chemicals used were of the highest available purity.

6.4.2.2 Animals

Adult male Wistar rats were used in this study and were housed and maintained as described in appendix one

6.4.2.3 Drug treatment

Rats were treated as described in section 4.1.2.3. However, on the 8^{th} day, two hours prior to killing, all animals received an intraperitoneal injection of 300 mg/kg of sodium salicylate. At 120 minutes following the salicylic acid injection rats were sacrificed by cervical dislocation, followed by decapitation. The striatum of each rat was dissected out from each hemisphere of the whole brain, frozen in liquid nitrogen and stored at -70 $^{\circ}$C.

6.4.2.4 Preparation of tissue for HPLC analysis

On the day of the experiment, the striatal samples were thawed on ice, weighed and sonicated (50 Hz for 60 s) in ice cold 0.1 M $HClO_4$ (1 mg of tissue in 10 µl). The sonicated samples were then centrifuged at 10 000 x g for 15 minutes using a bench top centrifuge. The supernatant (20 µl) was injected directly into the HPLC-ECD system for analysis.

6.4.2.5 Instrumentation

As described in section 2.2.2.3

6.4.2.6 Chromatographic conditions

Separation was achieved using an Ultrasphere C18 IP 80A analytical column (5 µm, 250 x 4.6 mm). The mobile phase consisted of an aqueous solution of 0.1 M sodium acetate trihydrate, 6 % methanol, 19.5 mg/L n-octyl sodium sulfate and 15 mg/L of EDTA (Sloot and Gramsbergen, 1995) and was degassed twice using a 0.45 µm membrane filter prior to use. The flow rate was 0.8 ml/min, and the electrodetection was performed at 0.65 V (Sloot and Gramsbergen, 1995).The amounts of methanol and n-octyl sodium sulfate were critical for achieving good separation and needed at least 8 hours (or overnight) equilibration with the columns by recycling the mobile phase.

6.4.2.7 Statistical analysis

As described in section 2.3.2.5.

6.4.3 RESULTS

Figure 6.12 shows the separation of 2,5-DHBA, 2,3-DHBA and CAT from the neurotransmitters NE, DA and 5-HT and their metabolites DOPAC, HVA and 5-HIAA.

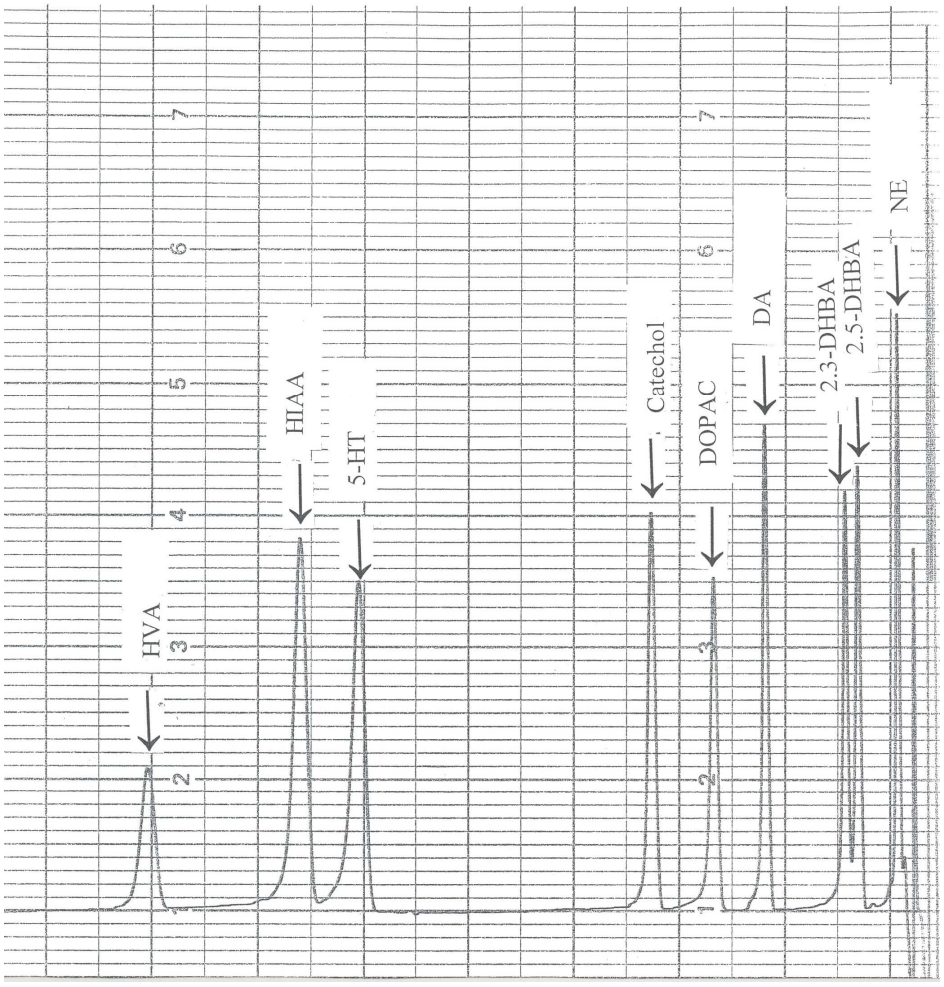


Figure 6.12: Chromatogram showing the separation of 2 pmol of external standards, including NE, 2,5-DHBA, 2,3-DHBA, DA, DOPAC, CAT, 5-HT, 5-HIAA and HVA using electrochemical detection at 0.65 V. Salicylic acid was not oxidized at this potential.

Table 6.1 shows the concentrations of 2,5-DHBA and 2,3-DHBA in striatal samples after the control and drug treated rats were injected i.p. with sodium salicylate (300 mg/kg) and killed 120 minutes later. CAT was not detected in the striatal tissues.

Table 6.1: Striatal concentrations of 2,3-DHBA and 2,5-DHBA following intraperitoneal administration of salicylic acid in saline, LD, SEL and (LD + SEL) pretreated rats.

Treatment (Twice daily)	2,5-DHBA (pmol/g)	2,3-DHBA (pmol/g)
Saline (control)	128.8 ± 24.5	36.5 ± 11.1
LD (10 mg/kg)	107.7 ± 26.7	26.3 ± 6.1
SEL (2.5 mg/kg)	103.9 ± 28.2	28.7 ± 8.9
LD (10 mg/kg) + SEL(2.5 mg/kg)	98.5 ± 26.4	25.4 ± 10.4

Concentrations of 2,3-DHBA and 2,5-DHBA did not differ significantly among the four treatment groups studied.

6.4.4 DISCUSSION

Striatal concentrations of 2,5-DHBA were higher than those of 2,3-DHBA, as expected (Althaus *et al*., 1995; Camp *et al*., 2000). The reason for this difference can most probably be attributed to the formation of 2,5-DHBA in the periphery by hydroxylation of salicylic acid by the cytochrome P450 system. The 2,5-DHBA formed in this way can then be transported to the brain via the circulatory system (Halliwell *et al*., 1991).

The results of the study indicate that the acute administration of LD does not result in a detectable increase in striatal •OH production *in vivo*. The results of the study contrast with those of a previous study (Smith *et al*., 1994), which found that the production of •OH in the *substantia nigra* was enhanced by the acute administration of high doses of

LD (200 mg/kg). It must be mentioned however that the doses of LD used in this study were extremely large compared to the average dose of LD administered to PD patients (approximately 10 mg/kg/day). A microdialysis study by Obata and Yamanaka (1996) also reported an elevation of both 2,3-DHBA and 2,5-DHBA when LD (0.1 mM) was infused in the rat brain through a microdialysis probe. However, a subsequent microdialysis study could not confirm these results (Camp *et al*., 2000). Except for the two studies mentioned above there are no other published reports indicating that the systemic administration of LD results in increased •OH formation in the striatum of nonlesioned animals; there are however numerous studies to the contrary (Loeffler *et al*., 1994; Ogawa *et al*., 1994; Camp *et al*., 2000).

LD is capable of producing H_2O_2 and reactive oxygen species by decarboxylation by L-amino acid decarboxylase to DA and subsequent oxidative deamination of DA to DOPAC and HVA by MAO or by the auto-oxidation of DA (Graham *et al*., 1978). SEL, would inhibit the formation of H_2O_2 by inhibiting the breakdown of DA and also has direct free radical scavenging activity (as demonstrated in the previous experiment). However, the present study shows that SEL is unable to suppress •OH formation below control levels. The reason for this may be that the endogenous antioxidant systems already present in the striatum may have masked the antioxidant effect of SEL.

As observed in chapter 4, the dose of LD used in this study would be expected to increase DA content in the striatum. If an increase in DA concentration and turnover produce an oxidative stress, as suggested by some studies (Cohen and Spina, 1989), then an increase in 2,3-DHBA in the present study could have been expected. A possible explanation as to why LD treatment did not result in increased striatal 2,3-DHBA and 2,5-DHBA is that normal striatal tissue may possess sufficient antioxidant defense mechanisms to prevent a rise in •OH levels. However, a study by Camp *et al*. (2000) reported that levels of 2,5-DHBA and 2,3-DHBA did not increase after acute or repeated LD administration (50 mg/kg) in rats with a 6-OHDA denervated striatum, indicating that even after severe damage, the striatum retains sufficient defense capacity to prevent an increase in •OH formation. A variety of other factors must also be taken into account in the treatment of

PD with LD. It is possible that other defects associated with PD, such as reduced mitochondrial enzyme complex I activity, GSH deficiency and increased concentrations of iron in the *substantia nigra* and the striatum (Fahn and Cohen, 1992; Jenner and Olanow, 1998) may increase the likelihood that an increase in DA levels induced by chronic LD therapy would result in oxidative stress. Another important consideration is that there are higher concentrations of iron in the nigrostriatal DA neurons in humans, as opposed to rats (Connor and Benkovic, 1992).

Although the results of this study suggest that LD does not increase formation of •OH in rat striatal tissue, LD may be toxic owing to other mechanisms. For example, the toxicity of LD and DA *in vitro* is thought to be due, in part to the production of toxic quinones and semiquinones formed during the auto-oxidation of these molecules. LD may also cause oxidative damage by depleting GSH (Spencer *et al*., 1995), potentiating excitotoxicity (Olney *et al*., 1990), or by inhibiting mitochondrial respiration (Cooper *et al*., 1995).

Other mechanisms by which LD may exert a neurotoxic effect, besides •OH production will be investigated in future chapters.

CHAPTER SEVEN

LIPID PEROXIDATION STUDIES

7.1 INTRODUCTION

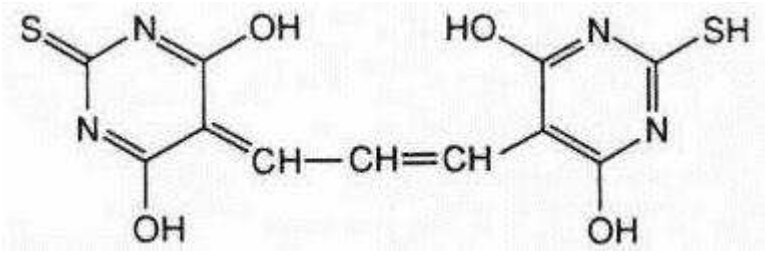
Biological membranes are vital to the life of cells, including neurons. The plasma membrane surrounds the cell and maintains the essential differences between the cytosol and the extracellular environment. Inside the cell the membranes of the endoplasmic reticulum, mitochondria and other membrane bounded organelles maintain the characteristic differences between the contents of these organelles and the cytosol. Membranes are dynamic, fluid structures that have a variety of different functions. Membranes function as permeable barriers for the selective transport of molecules into and out of the cell, are responsible for the production of ATP and also contain receptors for the binding of regulatory molecules such as hormones, growth factors and in the case of neurons, neurotransmitters to produce and transmit electrical signals. The mechanical properties of membranes are equally remarkable. When the cell grows or changes shape, the cell membrane can deform without tearing and addition of new membrane can occur without membrane ever losing its continuity. If the membrane is pierced, it does not collapse or remain torn but quickly reseals itself (Alberts *et al*., 1994).

An important target of free radicals is the polyunsaturated fatty acids (PUFA) in biological membranes. The peroxidation of these polyunsaturated lipids is known as lipid peroxidation and an important aspect of the process of lipid peroxidation is that it is self-perpetuating and will proceed until all the substrate is consumed or until termination occurs (Montine *et al*., 2002). Lipid peroxidation may impair membrane functions, increase ion permeability, reduce the membrane fluidity, inhibit the signal transduction over the membrane and inactivate membrane-bound enzymes and receptors (Bast *et al*., 1991). Electrophilic lipid peroxidation products such as 4-hydroxy-2-nonenal (HNE) has also been shown to covalently modify proteins (Camandola *et al*., 2000), block

mitochondrial respiration (Picklo *et al*., 1999) and induce caspase dependent apoptosis (Liu *et al*., 2000).

In the previous two chapters, DA and 6-OHDA were shown to increase the formation of $O_2^{\bullet-}$ and •OH *in vitro*. In addition to this the systemic administration of LD was shown to increase $O_2^{\bullet-}$ production *in vivo*. The generation of these free radicals, especially •OH may initiate the process of lipid peroxidation. Thus, this series of experiments investigates the effect of DA and 6-OHDA on iron (II) induced lipid peroxidation *in vitro* and the effect of systemic administration of LD on iron (II) induced lipid peroxidation *in vivo*. The previous experiments have also shown that SEL is able to reduce $O_2^{\bullet-}$ and •OH formation. This chapter will therefore also investigate the ability of SEL to protect lipids against oxidative damage induced by ROS.

The thiobarbituric acid (TBA) assay was used to investigate the capacity of DA and 6-OHDA to enhance iron (II) induced lipid peroxidation. The assay involves the reaction of malondialdehyde (MDA), an end product of lipid peroxidation with two molecules of TBA to yield a pink MDA-TBA chromagen (figure 7.1) that absorbs light at 532 nm and is readily extractable into organic solvents such as butanol (Halliwell and Gutteridge, 1989)



MDA-TBA complex

Figure 7.1: The structure of the pink MDA-TBA complex.
(http://www.genprice.com/tbars.htm)

7.2 EFFECT OF DOPAMINE AND 6-HYDROXYDOPAMINE ON IRON (II)-INDUCED LIPID PEROXIDATION IN RAT BRAIN HOMOGENATE *IN VITRO*

7.2.1 INTRODUCTION

The auto-oxidation and MAO mediated metabolism of DA and 6-OHDA both involve the generation of H_2O_2, a compound that can easily be reduced to form •OH in the presence of Fe^{2+}. This radical is considered to be the most damaging free radical for living cells and results in the deleterious oxidation of important biomolecules. A potentially devastating effect of •OH on membrane lipids is its ability to initiate the process of lipid peroxidation by abstracting a hydrogen atom from a PUFA of a membrane lipid (Halliwell, 1992). The previous chapter showed that DA can enhance the production of •OH by the Fenton system (Fe(II)-EDTA/H_2O_2). Chapter 2 also demonstrated that •OH can convert DA to 6-OHDA. Studies have shown that 6-OHDA also enhances •OH production by the Fenton system (Méndez-Álvarez *et al*., 2001).

The catalytic effect of iron to induce the auto-oxidation of DA and 6-OHDA and to form ROS has been related to the pathogenesis of PD (Kienzl *et al*., 1995; Linert *et al*., 1996). This hypothesis is strengthened by the increased risk of developing PD in patients who are exposed to transition metals during their occupation (Wang *et al*., 1989; Seidler *et al*., 1996) and the increased levels of iron in the SN of parkinsonian patients (Hirsch *et al*., 1991).

Postmortem investigations have consistently shown an increase in lipid peroxide levels in the brains of parkinsonian patients (Dexter *et al*., 1989a; Jenner *et al*., 1992). In addition to this, levels of HNE, an aldehyde formed during the lipid peroxidation process is increased approximately 6-fold in the SN of PD brains compared to age matched control

brains (Zhang *et al*., 1999). The present study therefore sought to investigate the effect of DA and 6-OHDA on lipid peroxidation induced by •OH formed during the Fenton reaction.

7.2.2 MATERIALS AND METHOD

7.2.2.1 Chemicals and reagents

DA hydrochloride, 6-OHDA hydrobromide, 2-thiobarbituric acid (TBA), 1,1,3,3-tetramethoxypropane and butylated hydroxytoluene (BHT) were purchased from Sigma Chemical Corporation, St Louis, MO, U.S.A. Trichloracetic acid (TCA) was purchased from Saarchem, Johannesburg, South Africa. EDTA and ferrous sulfate were purchased from Merck, Darmstadt, Germany. All other chemicals were of the highest quality available and were purchased from commercial distributors.

Stock solutions of DA and 6-OHDA were prepared in 1 mM KCl (pH 2.0) to prevent their immediate auto-oxidation. Fresh stock solutions of Fe^{2+} were prepared in deaerated water immediately before each experiment to prevent oxidation of the Fe^{2+} to Fe^{3+}. BHT (0.5 mg/ml) was dissolved in methanol. TBA (0.33 % m/v) was dissolved in Milli-Q water and protected from light by covering the solution with foil. TCA (15 % m/v) was dissolved in Milli-Q water.

7.2.2.2 Preparation of the standard curve

The construction of a calibration curve for this assay is complicated by the fact that MDA is very unstable and must therefore be prepared immediately before used by hydrolyzing its derivatives 1,1,3,3-tetramethoxypropane or 1,1,3,3-tetraethoxypropane (Halliwell and Gutteridge, 1989). In this case, 1,1,3,3-tetramethoxypropane was used to prepare the standard curve. A series of reaction tubes, each containing appropriate concentrations of 1,1,3,3-tetramethoxypropane (5 – 50 nmol/ml at 5 nmol/ml intervals) dissolved in PBS

was prepared. The procedure described in section 7.2.2.6 was then followed. The absorbance of the pink butanol fraction was then measured at 532 nm using a Shimadzu UV-160A UV-visible recording spectrophotometer. A calibration curve of absorbance versus concentration was then plotted (appendix 4).

7.2.2.3 Animals

Animals were housed and maintained as described in appendix one.

7.2.2.4 Brain removal

Rats were sacrificed by cervical dislocation, followed by decapitation, and the brains were rapidly removed for use in experiments as described in appendix two. The brains were either used immediately or stored at -70 ºC until needed.

7.2.2.5 Homogenate preparation

Each brain was weighed and homogenized (10 % m/v) in ice cold 0.1 M PBS, pH 7.4 in a glass teflon homogenizer. This is necessary to prevent lysosomal damage to the tissue. The homogenate was then used immediately for the assay.

7.2.2.6 Lipid peroxidation assay

A modification of the method of Placer *et al*. (1966) was used in this experiment.

Rat brain homogenate (0.4 ml) was incubated at 37 ºC for 5 minutes to reach the working temperature. Then, hydrogen peroxide (100 µM), ascorbate (100 µM), EDTA (240 µM) and ferrous sulfate (200 µM) were incorporated into the incubation (final volume 1 ml) and the mixture incubated for 1 hour in an oscillating water bath at 37 ºC. To investigate

the effect of DA and 6-OHDA on lipid peroxidation various concentrations of DA or 6-OHDA (0.05, 0.1, 0.15, 0.20 and 0.25 mM) were added to the incubation before the addition of the ferrous sulfate. At the end of the incubation period 0.5 ml of BHT (0.5 mg/ml) and 1 ml of TCA (15 % m/v) was added to prevent the further amplification of lipid peroxidation during the assay. The tubes were sealed and heated for 15 minutes in a boiling water bath to release protein-bound malondialdehyde (MDA). To avoid adsorption of MDA onto insoluble protein, the samples were cooled and centrifuged at 2000 x g for 15 minutes. Following centrifugation, 2 ml of the protein free supernatant was removed from each tube and 0.5 ml of TBA (0.33 % m/v) was added to this fraction. The resulting mixture was then incubated at 95 ºC in an acidic pH for 1 hour. After cooling to room temperature, 2 ml of butanol was added and the resulting mixture was shaken vigorously. After centrifugation at 2000 x g for 5 minutes the supernatant (butanol layer) was measured at 532 nm using a Shimadzu UV-160A UV-visible recording spectrophotometer. For calibration, a calibration curve (as described in section 7.2.2.2) was generated using the MDA derived from the acid hydrolysis of 1,1,3,3-tetramethoxypropane and the results are expressed as nmol MDA/mg of tissue.

7.2.2.7 Statistical analysis

Results were analysed as described in section 2.3.2.5.

7.2.3 RESULTS

As shown in figure 7.2 the analysis of MDA levels revealed that the incubation of whole rat brain homogenate at 37 ºC for 1 hour in the presence of the Fenton system (Fe(II)-EDTA/H_2O_2 and ascorbate) provokes a marked increase in MDA levels compared to levels observed in control incubations (approximately a 16-fold increase in MDA levels). The presence of either DA or 6-OHDA in the incubation with the Fenton system caused a significant and concentration dependent decrease in the MDA content of the rat brain homogenate. Both the 0.25 mM concentration of DA and 6-OHDA resulted in an

approximate 8-fold decrease in MDA levels. When DA and 6-OHDA (0.25 mM) were added to the incubation in the absence of the Fenton system, no significant increase in MDA was detected compared to the control values.

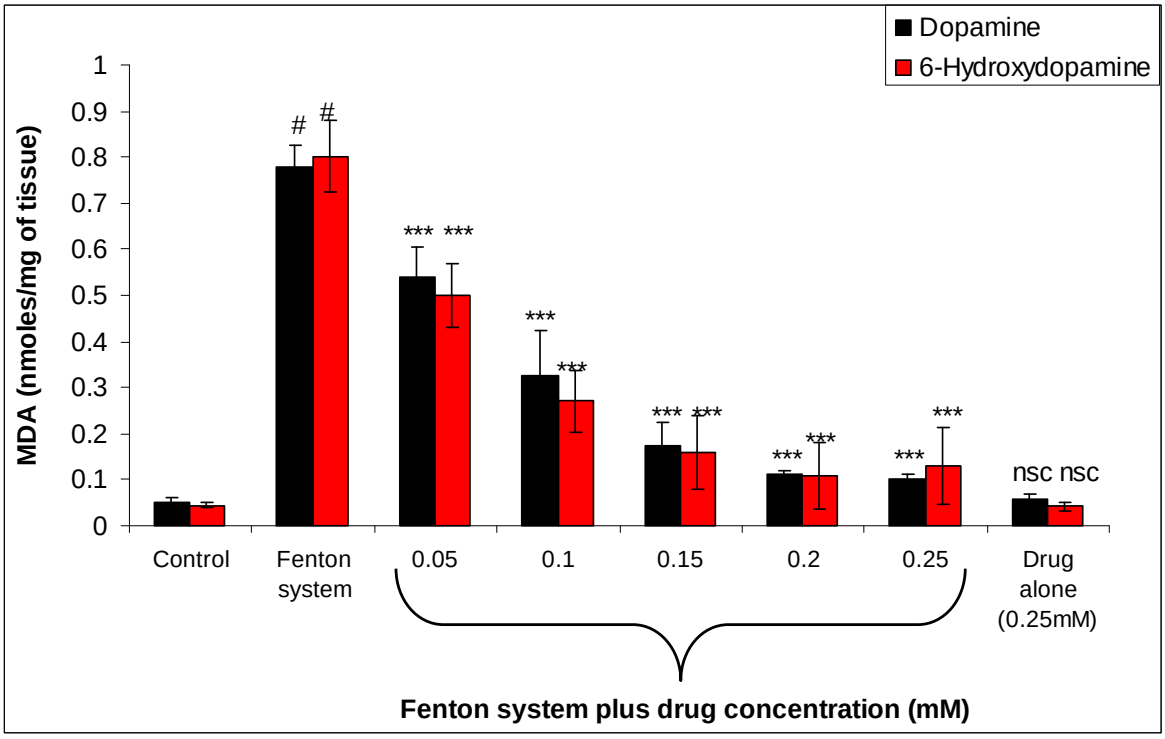


Figure 7.2: Effect of DA and 6-OHDA on lipid peroxidation in the presence and absence of the Fenton system. Each bar represents the mean ± SD (n = 5). nsc ($p > 0.05$) and # ($p < 0.001$) compared to control values; *** ($p < 0.001$) compared to the Fenton system group.

7.2.4 DISCUSSION

The results of the present study show that the addition of Fe(II)-EDTA/H_2O_2 and ascorbate (Fenton system) to whole rat brain homogenate provokes a marked increase in MDA levels. This is most likely due to the formation of the highly reactive •OH radical via the Fenton reaction. Iron can also participate in the formation of the hydroperoxyl radical (HOO•) as follows:

$Fe^{2+} + O_2 \rightarrow Fe^{3+} + O_2^{\bullet-}$ (Equation 7.1)

$O_2^{\bullet-} + H^+ \leftrightarrow HOO\bullet$ (Equation 7.2)

In solution, $O_2^{\bullet-}$ exists in equilibrium with the hydroperoxyl radical (HOO•), a far more reactive and lipid soluble radical than $O_2^{\bullet-}$ (Reiter, 1998). In addition to this HOO• has a higher rate of dismutation to H_2O_2 than $O_2^{\bullet-}$. The increased formation of H_2O_2 may therefore also contribute to the generation of •OH via the Fenton reaction. Both •OH and HOO• are believed capable of initiating the process of lipid peroxidation by abstracting a hydrogen atom from an unsaturated lipid (LH) giving rise to a lipid radical (L•, Equation 7.3) (Aikens and Dix, 1991; Gebicki and Gebicki, 1993). The lipid radical (L•) reacts with oxygen to form a peroxyl radical (LOO•, Equation 7.4). The chain of lipid peroxidation is sustained by the abstraction of hydrogen atoms from adjacent lipids by LOO•, leaving behind a carbon centered radical and a lipid hydroperoxide (LOOH) (Equation 7.5).

Lipid peroxidation reactions

$LH + \bullet OH \rightarrow L\bullet + H_2O$ **OR** $LH + HOO\bullet \rightarrow L\bullet + H_2O$ (Equation 7.3)

$L\bullet + O_2 \rightarrow LOO\bullet$ (Equation 7.4)

$LOO\bullet + LH \rightarrow LOOH + L\bullet$ (Equation 7.5)

The present study shows that DA and 6-OHDA result in a significant and concentration dependent decrease in lipid peroxidation stimulated by the Fenton system. The results of

this study corroborate previous studies showing that catecholamines such as DA, NE and 5-HT inhibit iron as well as iron-plus-ascorbate mediated lipid peroxidation in rat brain homogenate (Zaleska and Floyd, 1985; Dostert *et al*., 1991), liposomes (Spencer *et al*., 1996), linoleic acid (Liu and Mori, 1993) and mitochondria (Liu and Mori, 1993). Some of these investigators postulate that the inhibition of lipid peroxidation by these catecholamines is due to both their ability to scavenge free radicals (particularly the scavenging of $O_2^{\bullet-}$ and •OH) and to chelate iron, thereby slowing or preventing iron participation in the Fenton reaction. However, the previous two chapters contradict this hypothesis since they demonstrate that both DA and 6-OHDA have the ability to generate $O_2^{\bullet-}$ and •OH during their metabolism and auto-oxidation. The generation of $O_2^{\bullet-}$ could also result in an increase in the generation of HOO• and •OH ($O_2^{\bullet-}$ providing the H_2O_2 necessary for Fenton chemistry and by reducing Fe^{3+} to Fe^{2+}). It is therefore likely that DA and 6-OHDA inhibit lipid peroxidation via a different mechanism which does not involve the direct scavenging of the above mentioned free radicals.

The most probable explanation is that the semiquinone radicals (SQ•) produced during the auto-oxidation of DA (figure 7.3) and 6-OHDA may act as chain breaking antioxidants by reacting with lipid radicals and preventing the propagation of lipid peroxidation as follows:

L• + •SQ → LH + Quinone (Equation 7.6)

LO• + •SQ → LOH + Quinone (Equation 7.7)

LOO• + •SQ → LOOH + Quinone (Equation 7.8)

Termination of the free radical chain reaction of lipid peroxidation occurs when two free radicals destroy each other.

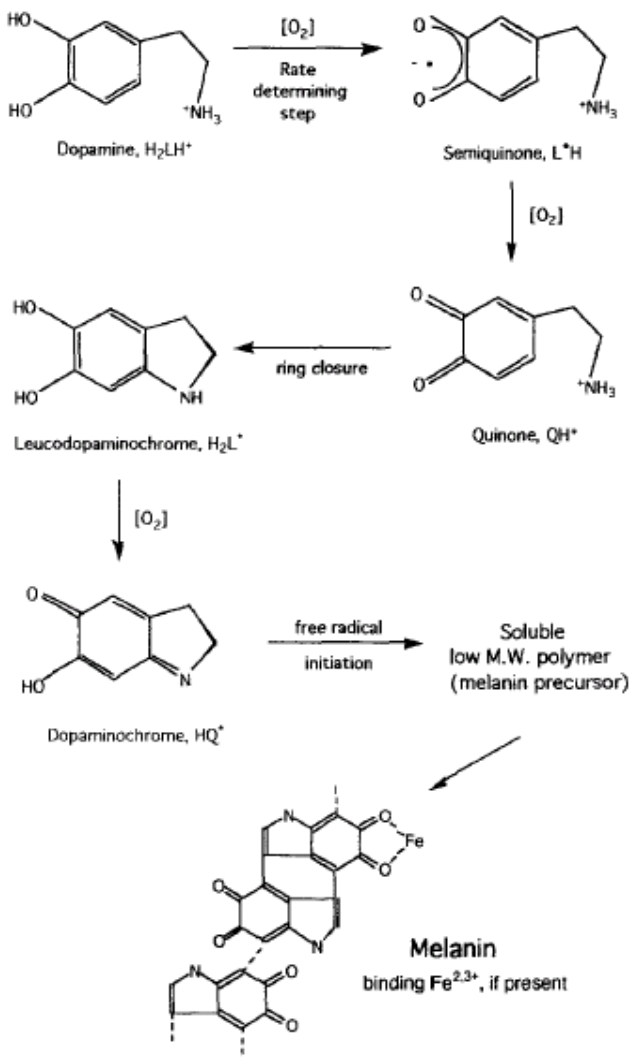


Figure 7.3: Mechansim of DA auto-oxidation showing the formation of a semiquinone radical in the rate limiting step (Linert *et al*., 1996).

The hypothesis that the inhibition of lipid peroxidation by DA may be a consequence of the capacity of SQ• to prevent the propagation of lipid peroxidation has also been proposed by Hermida-Ameijeiras *et al*. (2004).

7.3 EFFECT OF SELEGILINE ON IRON (II)-INDUCED LIPID PEROXIDATION IN RAT BRAIN HOMOGENATE *IN VITRO*

7.3.1 INTRODUCTION

The previous two chapters show that SEL has the ability to reduce the formation of both $O_2^{\bullet-}$ and •OH. It is well established that •OH produced in free solution can initiate the lipid peroxidation process by hydrogen abstraction. There is also no reason why $O_2^{\bullet-}$ dependent Fenton chemistry should not initiate the process of lipid peroxidation in the same way ($O_2^{\bullet-}$ providing the H_2O_2 necessary for Fenton chemistry and by reducing Fe^{3+} to Fe^{2+}). In addition to this, $O_2^{\bullet-}$ can be protonated to form HOO• which has also been shown to initiate lipid peroxidation in purified fatty acid (Wills, 1966). By reducing the formation of $O_2^{\bullet-}$ and •OH, SEL should therefore be able to inhibit lipid peroxidation stimulated by Fenton chemistry.

The main aim of this investigation is to determine whether SEL can inhibit Fenton stimulated lipid peroxidation in rat brain homogenate on its own and whether it can enhance the inhibition of lipid peroxidation by DA (observed in the previous study).

7.3.2 MATERIALS AND METHOD

7.3.2.1 Chemicals and reagents

SEL hydrochloride was purchased from Sigma Chemical Corporation, St Louis, MO, U.S.A. All other reagents were purchased as described in section 7.2.2.1

7.3.2.2 Preparation of the standard curve

As described in section 7.2.2.2

7.3.2.3 Animals

Animals were housed and maintained as described in appendix one.

7.3.2.4 Brain removal

As described in section 7.2.2.4

7.3.2.5 Homogenate preparation

As described in section 7.2.2.5

7.3.2.6 Lipid peroxidation assay

A modification of the method of Placer *et al*. (1966) was used in this experiment.

The lipid peroxidation assay was carried out as described in section 7.2.2.6, except that various concentrations of SEL (0.25, 0.5, 0.75 and 1 mM) instead of DA or 6-OHDA were added to the incubation prior to the addition of the ferrous sulfate.

In some cases, various concentrations of DA (0.05, 0.1, 0.15, 0.2 and 0.25 mM) were incubated with either the lowest concentration (0.25 mM) or the highest concentration of SEL (1 mM) to investigate whether SEL could enhance the decrease in lipid peroxidation induced by DA.

7.3.2.7 Statistical analysis

Results were analysed as described in section 2.3.2.5.

7.3.3 RESULTS

Figure 7.4 shows that the incubation of rat brain homogenate with various concentrations (0.25, 0.5, 0.75 and 1 mM) of SEL led to a decrease in MDA levels in relation to the amount of MDA found in rat brain homogenate exposed to the Fenton system alone. The 1 mM concentration of SEL resulted in a ± 45 % decrease in MDA levels compared to the Fenton system group. However, it is also clear that higher concentrations of SEL are needed to inhibit Fenton stimulated lipid peroxidation compared to DA and 6-OHDA.

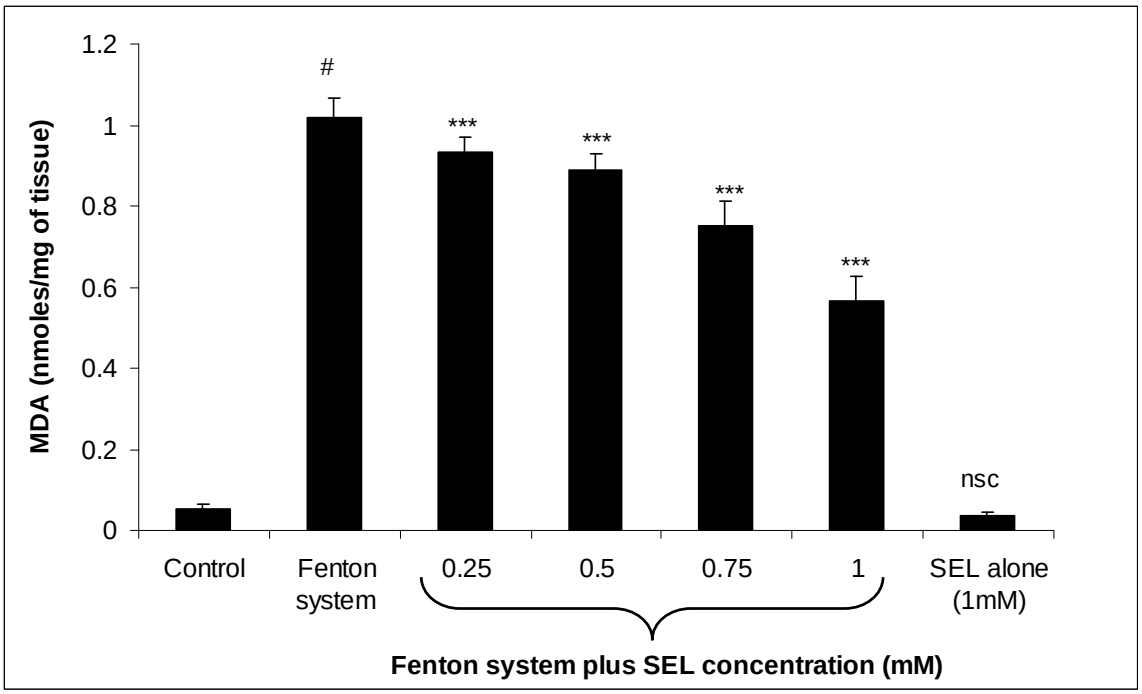


Figure 7.4: Effect of SEL on lipid peroxidation in the presence and absence of the Fenton system. Each bar represents the mean ± SD (n = 5). nsc ($p > 0.05$) and # ($p < 0.001$) compared to control values; *** ($p < 0.001$) compared to the Fenton system group.

Figure 7.5 shows that the incubation of rat brain homogenate with SEL (0.25 mM) enhances the ability of DA to inhibit lipid peroxidation induced by the Fenton system when DA is used at relatively low concentrations of 0.05 and 0.1 mM. However, when higher concentrations of DA (0.15, 0.2 and 0.25 mM) were used in combination with SEL (0.25 mM), the addition of SEL did not alter the MDA values obtained with the use of DA alone.

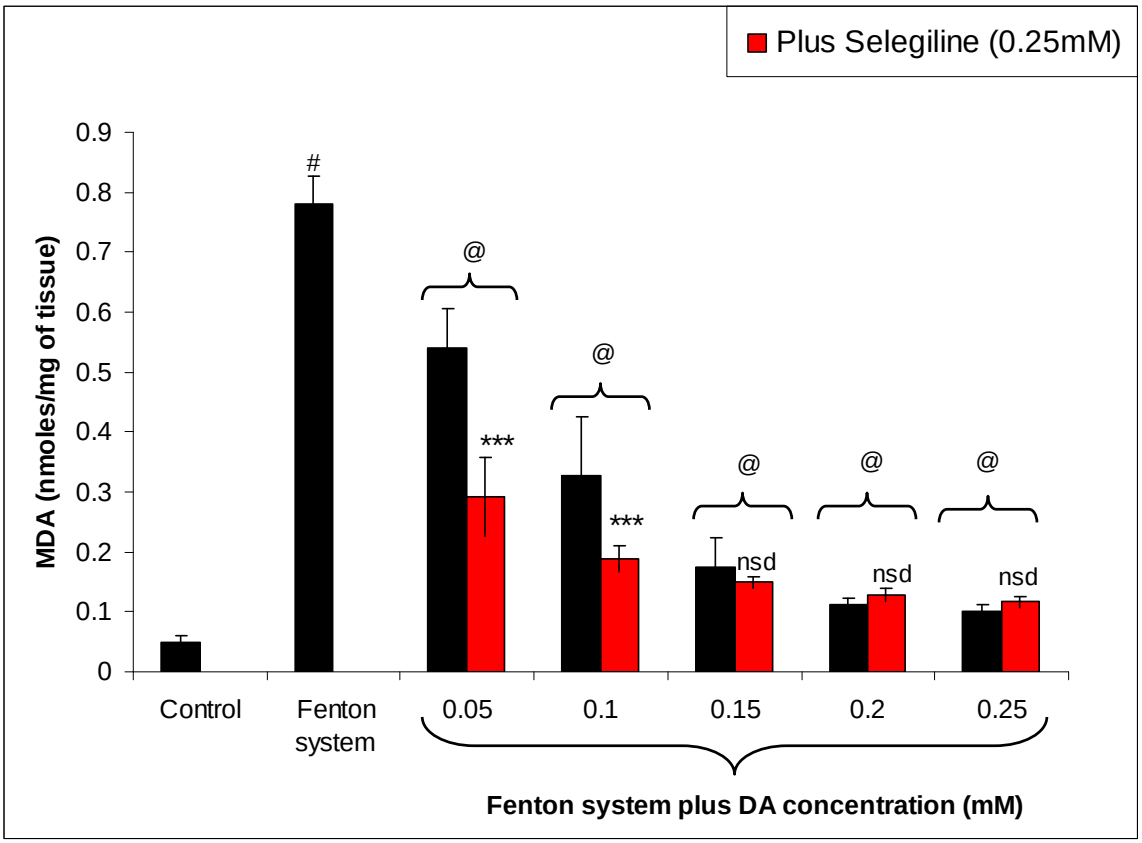


Figure 7.5: Combined effect of DA and SEL (0.25 mM) on lipid peroxidation in the presence of the Fenton system. Each bar represents the mean ± SD (n = 5). # ($p < 0.001$) compared to control values; @ ($p < 0.001$) compared to the Fenton system group; *** ($p < 0.001$) and nsd ($p > 0.05$) compared to the corresponding DA group.

Figure 7.6 shows that the combined action of relatively low concentrations of DA (0.05 and 0.1 mM) and SEL (1 mM) results in a greater inhibition of Fenton stimulated lipid peroxidation than the use of DA (0.05 and 0.1 mM) or SEL (1 mM) alone. The results

were therefore similar to those obtained with the lower concentration of SEL (0.25 mM) shown in figure 7.5. Curiously, the ability of SEL to amplify the inhibition of Fenton stimulated lipid peroxidation by DA is lost when using higher concentrations of DA (0.15, 0.2 and 0.25 mM). In this case, the combined action of DA (0.15, 0.2 or 0.25 mM) and SEL (1 mM) results in significantly ($p < 0.001$) higher levels of MDA than those obtained with the use of DA alone.

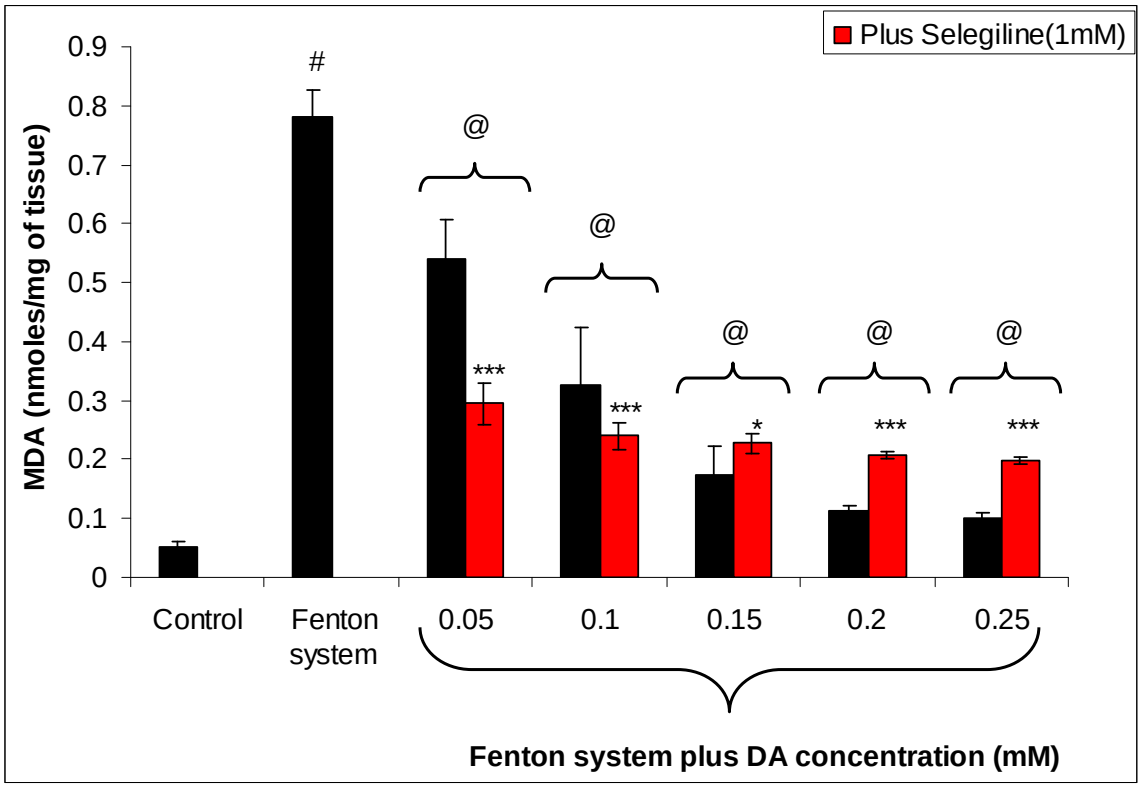


Figure 7.6: Combined effect of DA and SEL (1 mM) on lipid peroxidation in the presence of the Fenton system. Each bar represents the mean ± SD (n = 5). # ($p < 0.001$) compared to control values; @ ($p < 0.001$) compared to the Fenton system group; *** ($p < 0.001$) and * ($p < 0.05$) compared to the corresponding DA group.

7.3.4 DISCUSSION

As described in the previous experiment, the incubation of rat brain homogenate with the Fenton system under physiological conditions of temperature and pH provokes a significant increase in MDA levels. The present study shows that the addition of increasing concentrations of SEL to incubations containing the Fenton system results in a significant decrease in lipid peroxidation. This decrease in lipid peroxidation is dependent on the concentration of SEL used. However, lower concentrations of DA and 6-OHDA inhibit lipid peroxidation more so than the relatively high concentrations of SEL used in this study. This therefore demonstrates that SEL is less effective than DA or 6-OHDA at inhibiting lipid peroxidation. The inhibition of lipid peroxidation by SEL is probably a consequence of the ability of SEL to inhibit the formation of toxic oxygen species such as $O_2^{\bullet -}$ and •OH that initiate the process of lipid peroxidation. SEL could also decrease the formation of H_2O_2 available to participate in the Fenton reaction by inhibiting the MAO-B catalyzed metabolism of endogenous monoamines (particularly DA) present in the rat brain homogenate.

The present study also shows that the addition of SEL to incubations containing the Fenton system and concentrations of DA below 0.1 mM enhances the ability of DA to inhibit lipid peroxidation. This is to be expected because SEL inhibits the formation of ROS formed during the auto-oxidation and MAO-B mediated metabolism of DA. However, a peculiar effect of SEL observed in this study is that when higher concentrations of DA were used (0.15 – 0.25 mM) the combined action of SEL and DA resulted in higher levels of MDA than those observed in the presence of DA alone. Higher concentrations of DA therefore appear to unmask a pro-oxidant effect of SEL. This phenomenon may be a consequence of the suggested capacity of •OH to abstract a hydrogen atom from the acetylenic MAO inhibitor SEL (Pryor, 1976) resulting in the formation of a carbon centered radical in the SEL molecule (SEL•). Therefore, during the scavenging of •OH SEL may be converted to SEL• (Equation 7.9), which in turn may eventually initiate the process of LP by abstracting a hydrogen atom from a membrane PUFA (Equation 7.10):

SEL-H + •OH → SEL• (Equation 7.9)

SEL• + LH → SEL-H + L• (Equation 7.10)

In this way the acetylenic group of SEL remains intact and SEL retains its MAO-B inhibiting activity (Kalir *et al*., 1981; Fowler *et al*., 1982; Hermida-Ameijeiras *et al*., 2004). At higher concentrations of DA, an increase in •OH formation by the Fenton system is expected to occur (as observed in the previous chapter). The scavenging of these •OH by SEL may then result in the accumulation of toxic levels of SEL• which may in turn initiate the lipid peroxidation process.

The next experiment will investigate the effect of systemic administration of LD and SEL, both separately and in combination with each other on striatal MDA levels.

7.4 EFFECT OF L-DOPA AND SELEGILINE TREATMENT ON IRON (II)-INDUCED LIPID PEROXIDATION IN THE RAT STRIATUM *IN VIVO*

7.4.1 INTRODUCTION

LD is the primary treatment for PD (LeWitt, 1989). Although the exact etiology of PD has not yet been unraveled there is increasing evidence linking it to elevated levels of ROS (Fahn and Cohen, 1992). Among the findings are reports of increased lipid peroxide levels in autopsy specimens of brains from parkinsonian patients (Dexter *et al*., 1989a; Jenner *et al*., 1992) as well as an increased amount of iron in the SN of PD patients (Dexter *et al*., 1991; Sofic *et al*., 1991). The intranigral infusion of ferrous citrate (1.3 - 8.4 nmol) results in an increase in lipid peroxidation in the SN, increases DA turnover in the caudate nucleus (Sziraki *et al*., 1998) and results in an elevation of tissue iron levels (Sengstock *et al*., 1993).

There has been concern that the use of LD in PD might contribute to the progression of PD by enhancing oxidative stress. However, Ogawa *et al*. (1994) reported that chronic LD treatment does not increase lipid peroxidation in the striatum of normal (intact) animals. Similarly, Dostert *et al*. (1991) failed to detect enhanced lipid peroxidation in rats treated with LD. In contrast, chronic administration of LD to animals with a lesioned nigrostriatal DA system was found to increase lipid peroxides in the rat striatum in some studies (Ogawa *et al*., 1994) but not others (Murer *et al*., 1998).

The previous studies in this chapter have shown that DA and even 6-OHDA inhibit Fe^{2+} induced lipid peroxidation. The main aim of this study is to investigate whether the i.p. administration of LD results in an increase or a decrease in the striatal lipid peroxidation induced by the infusion of ferrous sulfate (10 nmol) into the striatum of male Wistar rats. The study also investigates the effect of i.p. SEL administration, on its own and in

combination with LD on the lipid peroxidation induced by the intrastriatal infusion of Fe^{2+}.

7.4.2 MATERIALS AND METHODS

7.4.2.1 Chemicals and reagents

L-3,4-dihydroxyphenylalanine (LD), benserazide hydrochloride and SEL hydrochloride were purchased from Sigma Chemical Corporation, St Louis, MO, U.S.A. All other chemicals and reagents were purchased as described in section 7.2.2.1.

7.4.2.2 Animals

Animals were housed and maintained as described in appendix one.

7.4.2.3 Drug treatment

Animals were injected intrastriatally with 2 μl of ferrous sulfate (5 mM) and dosed with LD and SEL for 7 days as described in section 2.5.2.3.2. Control rats received an intrastriatal injection of physiological saline. On the morning after the last dose, rats were sacrificed by cervical dislocation, followed by decapitation. Brains were rapidly removed and the iron infused striata of each rat were dissected free and analysed for MDA.

7.4.2.4 Homogenate preparation

Each striatum was weighed and homogenized (5 % m/v) in ice cold 0.1 M PBS, pH 7.4. This is necessary to prevent lysosomal damage to the tissue. The homogenate was then used immediately for the assay.

7.4.2.5 Lipid peroxidation assay

A modification of the method of Placer *et al*. (1966) was used in this experiment. A calibration curve was constructed as described in section 7.2.2.2.

The procedure in section 7.2.2.6 was followed except that the incubation step at 37 ºC for 1 hour was excluded. In addition to this, due to the small size and weight of the striatum a smaller volume of homogenate was used for the assay (0.5 ml). Briefly, the homogenate (0.5 ml) for each treated group was heated for 15 minutes with 0.25 ml of BHT (0.05 %) and 0.5 ml of TCA (15 %) to release protein bound MDA. The procedure described in section 7.2.2.6 was then followed and results are expressed as MDA (nmol/mg of tissue).

7.4.2.6 Statistical analysis

Results were analysed as described in section 2.3.2.5.

7.4.3 RESULTS

Figure 7.7 shows that the injection of ferrous sulfate (10 nmol) into the rat striatum results in a significant increase in striatal MDA content ($p < 0.001$). However, levels of MDA in the Fe^{2+} + LD treated rats were significantly lower ($p < 0.05$) than in those rats that only received the intrastriatal injection of Fe^{2+}. The i.p. administration of LD caused a ± 27 % decrease in striatal MDA content relative to MDA levels measured in Fe^{2+} only treated rats. In the Fe^{2+} + SEL treated rats there was a ± 39 % decrease in MDA levels relative to the levels of MDA detected in the Fe^{2+} only treated rats ($p < 0.01$).

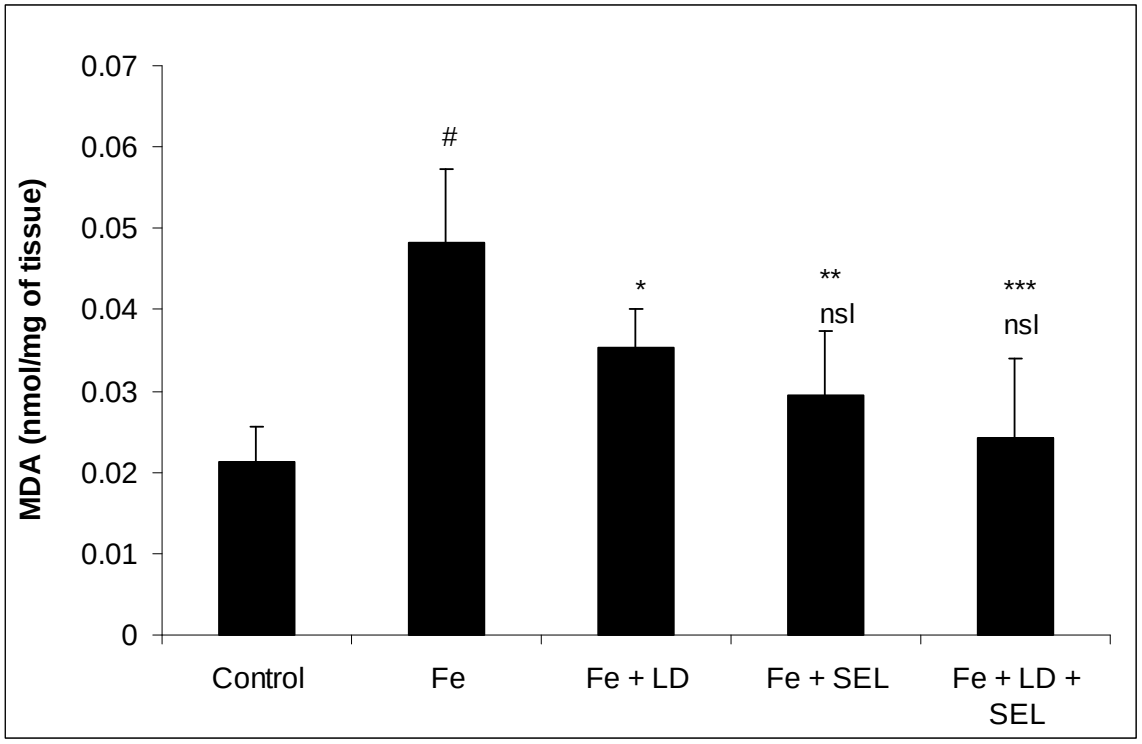


Figure 7.7: Effect of intrastriatal injections of ferrous sulfate (10 nmol) followed by LD and SEL treatment on rat striatal MDA content. Each bar represents the mean ± SD (n = 5). # ($p < 0.001$) compared to the control; * ($p < 0.05$), ** ($p < 0.01$) and *** ($p < 0.001$) compared to rats that received Fe only; nsl ($p > 0.05$) compared to the Fe + LD treatment group.

Thus, the i.p. injection of LD or SEL significantly inhibits lipid damage induced by an increase in striatal iron levels. It is also evident from figure 7.7 that the co-administration of LD and SEL results in an even more significant decrease in lipid peroxidation ($p < 0.001$) induced by elevated Fe^{2+} levels in the striatum than that observed when either DA ($p < 0.05$) or SEL ($p < 0.01$) is used alone. Despite this fact, no statistically significant difference in MDA levels was detected between the Fe + LD, Fe + SEL and the Fe + LD +SEL treatment groups.

7.4.4 DISCUSSION

Several areas of the brain are known to be rich in iron, a known catalyst of lipid peroxidation *in vitro* and *in vivo*. The iron content of the brain is highest in the *substantia nigra* and the striatum (*globus pallidus, caudate nucleus* and *putamen*) (Sengstock *et al*., 1993; Wesemann *et al*., 1994). Despite the fact that 90 % of the brains nonheme iron is stored in an inactive form bound to ferritin (Koeppen and Dentinger, 1988; Kaneko *et al*., 1989; Connor *et al*., 1990), the concentration of free intracellular Fe^{2+} in the brain is approximately 1 µM (Williams, 1982). Furthermore, biochemical studies and magnetic resonance imaging of parkinsonian brains have shown a significant elevation of iron in PD brains compared to age matched control brains (Dexter *et al*., 1989a; Drayer *et al*., 1986). The results of this study show that the intrastriatal injection of Fe^{2+} (10 nmol) results in a significant increase in lipid peroxidation in the rat striatum, a finding which is consistent with the results of Sengstock *et al*., (1993) and Sziraki *et al*., (1998) demonstrating an increase in lipid peroxidation and extensive damage to nigrostriatal neurons following intracerebral injections of ferrous iron.

The results of the present study also show that the i.p. administration of LD results in a significant decrease in Fe^{2+} stimulated lipid peroxidation. Once again, the inhibition of lipid peroxidation by LD appears to be a consequence of the suggested capacity of SQ• to block the propagation of lipid peroxidation and not due to the scavenging of •OH since LD has the ability to generate this free radical by decarboxylation to DA and subsequent auto-oxidation or MAO catalysed metabolism of DA. The i.p. administration of SEL caused a reduction in the striatal MDA content relative to the Fe^{2+} only group. This finding agrees with the ability of SEL to inhibit the formation of $O_2^{\bullet}$ (and consequently HOO•), H_2O_2 (by inhibiting the breakdown of DA) and •OH. Another interesting observation of the present study is that the co-administration of LD and SEL results in a more significant decrease ($p < 0.001$) in Fe^{2+} induced lipid peroxidation than LD ($p < 0.05$) or SEL ($p < 0.01$) alone. This result suggests that the co-administration of LD and SEL in the treatment of PD may offer better protection against lipid peroxidation and subsequent neuronal damage than the use of LD alone.

It has been suggested that catechol containing compounds can shift oxidative damage from lipid peroxidation to protein oxidation (Boots *et al*., 2002). The next chapter will therefore investigate the effect of DA, 6-OHDA and LD on protein oxidation.

CHAPTER EIGHT

PROTEIN OXIDATION STUDIES

8.1 INTRODUCTION

The introduction of carbonyl groups into the amino acid residues of proteins is a hallmark feature of protein oxidation. Carbonyl derivatives are formed by ROS mediated oxidation of side chains of some amino acid residues (Aksenov *et al*., 2001). Carbonyl groups can also be introduced into proteins by glycation and reactions with products of lipid peroxidation and glycosidation reactions (Aksenov *et al*., 2001). Reaction of these carbonyl groups with 2,4-dinitrophenyl hydrazine provides a method for detecting and quantifying the extent of protein oxidation.

A number of studies have shown that enzymes and proteins are damaged by exposure to DA. Two different mechanisms have been suggested to explain the oxidative damage to proteins induced by DA. In one proposed mechanism, toxic oxygen species such as H_2O_2, $O_2^{\bullet-}$ and •OH generated from the oxidation of DA are implicated in the protein damage (Halliwell, 1992; Basma *et al*., 1995; Dunnett and Bjorklund, 1999). In the second suggested mechanism, the catechol ring of DA undergoes oxidation to form various quinone products. DA quinone (DA-Q) can undergo intermolecular Michael addition reactions with protein bound nucleophiles, particularly cysteinyl residues (figure 8.1) and low molecular weight species e.g. GSH (Rotman *et al*., 1976; Ito *et al*., 1988; Basma *et al*., 1995). The latter reaction can result in the depletion of cellular GSH (Nappi and Vass, 1994) whereas the former reaction results in the covalent binding of the quinone to the protein (Ito *et al*., 1984). Since cysteinyl residues are often found in the active site of proteins, covalent addition of DA-Q to cysteine (Cys) may inhibit protein function and could possibly lead to cell damage or cell death.

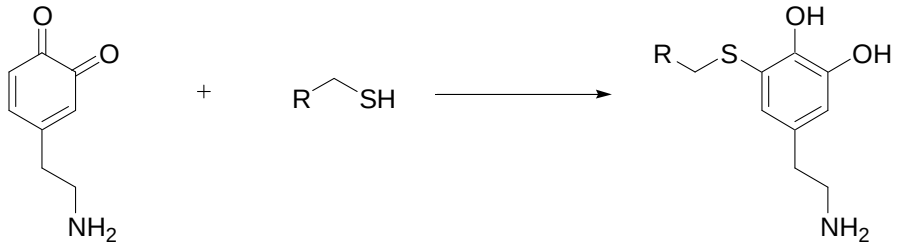


Figure 8.1: Reaction of DA-Q with thiol groups.

DA-Q can also undergo Schiff-base reactions with amine functions, particularly lysine (Lys) side-chains on proteins (figure 8.2), however the formation of these linkages appears to occur more slowly than with Cys (Ito *et al*., 1984).

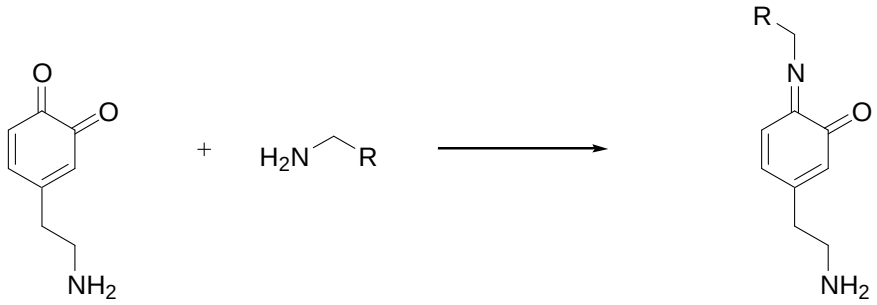


Figure 8.2: Reaction of DA-Q with amino groups.

Chapter 2 describes that in the presence of Fe(II)-EDTA/H_2O_2 and ascorbate (Fenton system), DA can be hydroxylated to form 6-OHDA. The conversion of DA to 6-OHDA may result in a significant increase in protein carbonyl content by enhancing the formation of both quinone products and reactive oxygen species.

This study sought to investigate the potential of DA to cause oxidative modification of proteins (increase in carbonyl content). The effects provoked by the presence of the Fenton system on protein oxidation caused by DA were also investigated in an attempt to assess whether the conversion of DA to 6-OHDA results in a significant increase in protein carbonyl content. Furthermore, SEL was used as a free radical scavenger in our system to establish whether the toxic oxygen species or the quinone products are responsible for the protein damage.

8.2 EFFECT OF DOPAMINE AND 6-HYDROXYDOPAMINE ON IRON (II)-INDUCED PROTEIN OXIDATION IN RAT BRAIN HOMOGENATE *IN VITRO*

8.2.1 INTRODUCTION

The oxidative modification of proteins attracts a great deal of interest in aging and age-related neurodegenerative diseases. Under conditions of oxidative stress, chemical transformations of amino acid residues can lead to a loss of protein function (Stadtman, 1990; Dean *et al*., 1997). Oxidized proteins are often converted to a form that is more susceptible to proteinases and oxidative modification can therefore "mark" proteins for degradation by proteolysis (Stadtman, 1990; Aksenov *et al*., 2001). Conversely, protein oxidation can also promote the formation of cross-linked protein aggregates that are resistant to degradation by proteinases (Butterfield and Stadtman, 1997).

The previous chapter showed that DA and 6-OHDA were able to inhibit hydroxyl free radical-induced lipid peroxidation. This was despite the fact that both DA and 6-OHDA enhance the generation of •OH by the Fenton system under similar conditions (chapter 6). It has been postulated that catechol containing compounds may shift oxidative damage from lipid peroxidation to protein oxidation (Boots *et al*., 2002). This may be a consequence of the reactive quinones formed during the auto-oxidation of these compounds.

This study therefore investigates the capacity of DA and 6-OHDA to cause protein oxidation (an increase in protein bound carbonyl groups). In addition, the study also investigates the effects provoked by the presence of the Fenton system on the extent of protein oxidation caused by DA and 6-OHDA.

8.2.2 MATERIALS AND METHODS

8.2.2.1 Chemicals and reagents

DA hydrochloride, 6-OHDA hydrobromide, 2,4-dinitrophenylhydrazine (2,4-DNPH) and guanidine hydrochloride were purchased from Sigma Chemical Corporation, St Louis, MO, U.S.A. TCA, ethanol and ethyl acetate were purchased from Saarchem, Johannesburg, South Africa. Streptomycin sulfate was purchased from Wallace's Pharmacy, Grahamstown. EDTA and ferrous sulfate were purchased from Merck, Darmstadt, Germany. All other chemicals were of the highest quality available and were purchased from commercial distributors.

Stock solutions of DA and 6-OHDA were prepared in 1 mM KCl (pH 2.0) to prevent their immediate auto-oxidation. Fresh stock solutions of Fe^{2+} were prepared in deaerated water immediately before each experiment to prevent oxidation of the Fe^{2+} to Fe^{3+}.

8.2.2.2 Animals

Animals were housed and maintained as described in appendix one.

8.2.2.3 Brain removal

Rats were sacrificed by cervical dislocation, followed by decapitation, and the brains were rapidly removed for use in experiments as described in appendix two. The brains were either used immediately or stored at -70 ºC until needed.

8.2.2.4 Homogenate preparation

Each brain was weighed and homogenized (10 % m/v) in ice cold 0.1 M PBS, pH 7.4 in a glass teflon homogenizer. The homogenate was then used immediately for the assay.

8.2.2.5 Protein carbonyl measurement

The protein carbonyl content was assessed spectrophotometrically according to a modified method of Levine *et al*. (1990).

Rat brain homogenates (10 % m/v in PBS) were diluted with PBS to 5 mg/ml of protein. Streptomycin sulfate (1 % final concentration) was then added to this and the homogenate was stirred gently for 15 minutes. The homogenate was then centrifuged at 11 000 x g for 10 minutes and the pellet was discarded. The supernatant was collected and diluted to 2.5 mg of protein/ml. Supernatant (2.5 mg of protein/ml) was incubated at 37 ºC for 5 minutes to reach the working temperature. Then, hydrogen peroxide (100 µM), ascorbate (100 µM), EDTA (240 µM) and ferrous sulfate (200 µM) were incorporated into the incubation and the mixture incubated for 60 minutes. To investigate the effect of DA and 6-OHDA on protein oxidation various concentrations of DA or 6-OHDA (0.25, 0.5, 0.75 and 1 mM) were added to the incubation before the addition of the ferrous sulfate. The final concentration of protein in the incubation after the addition of the above mentioned reagents was 1 mg/ml. Immediately after the incubation, protein precipitation was achieved with the addition of TCA (10 % final concentration) followed by centrifugation at 11 000 x g for 5 minutes. The resulting pellet was reconstituted in NaOH (0.5 M) with vigorous vortexing. Then, 0.5 ml of 2,4-dinitrophenylhydrazine (2 mM final concentration) was added and the mixture incubated at room temperature for 1 hour, in darkness and with vortexing every 10 – 15 minutes. Proteins were then precipitated by the addition of TCA and recovered after centrifuging at 11 000 x g for 10 minutes. The resulting pellet was washed twice with ethyl acetate: ethanol (1:1, v/v). The washed pellet was then reconstituted with 6 M guanidine and the absorbance of the

resulting solution measured at 370 nm. The carbonyl content was calculated using a molar absorption coefficient of 22 000 $M^{-1}cm^{-1}$. Results are expressed as nmol carbonyls/ mg of protein. Protein estimation was obtained from a protein standard curve (appendix five) using the method described by Lowry *et al*., (1951). Bovine serum albumin (BSA) was used for the construction of the calibration curve.

8.2.2.6 Statistical analysis

Results were analysed as described in section 2.3.2.5.

8.2.3 RESULTS

As illustrated in figure 8.3, the incubation of rat brain proteins with DA at 37 ºC for 1 hour causes an increase in the carbonyl content of the proteins. The addition of the Fenton system to the incubation in the absence of DA also results in a significant increase in carbonyl content of the proteins. However, the combined action of the Fenton system and DA significantly augmented the carbonyl content that was obtained in the presence of the Fenton system or DA alone. The addition of 6-OHDA to the protein incubations results in a significant increase in protein oxidation when compared to control values. As expected, the augmentation of carbonyl content observed with 6-OHDA was greater than that obtained with DA. Curiously, the combined action of the Fenton system and 6-OHDA did not increase the carbonyl content that was obtained in the presence of 6-OHDA alone.

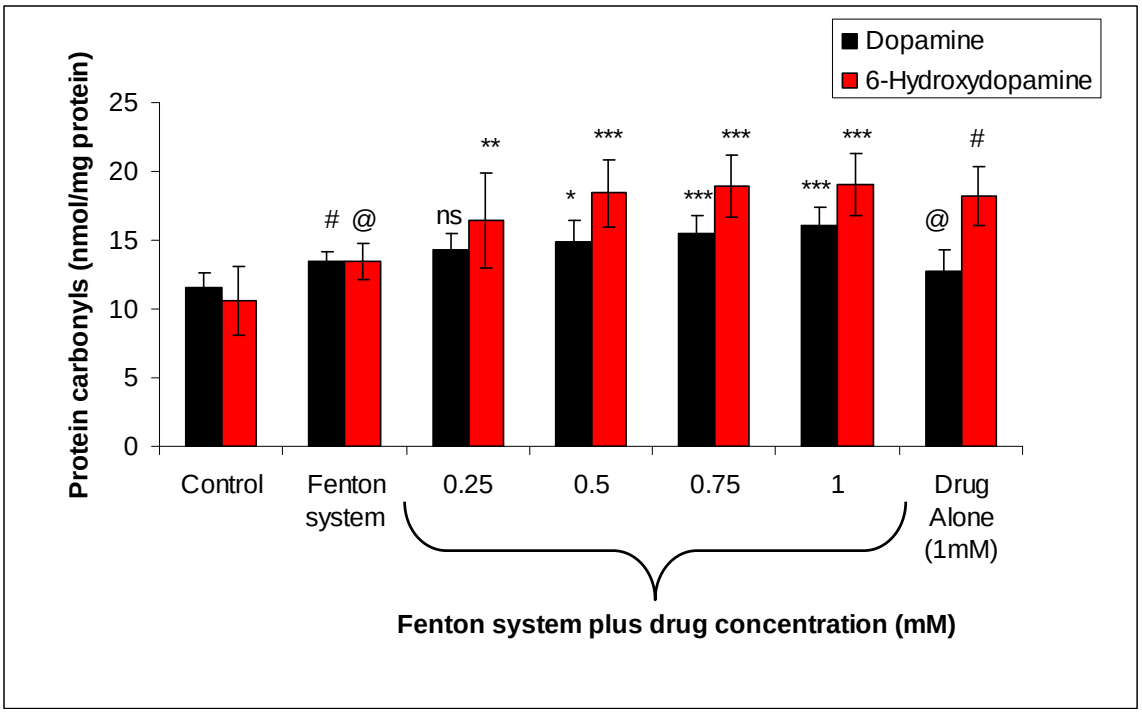


Figure 8.3: Effect of DA and 6-OHDA on protein oxidation in the presence and absence of the Fenton system. Each bar represents the mean ± SD (n = 5). @ ($p < 0.05$) and # ($p < 0.001$) compared to control values; ns ($p > 0.05$), * ($p < 0.05$), ** ($p < 0.01$) and *** ($p < 0.001$) compared to the Fenton system group.

8.2.4 DISCUSSION

To gain additional insight into the molecular mechanisms involved in the neurotoxicity of DA, the present study investigated the capacity of DA to cause protein oxidation in rat brain homogenate. Furthermore, in order to investigate the consequences of the conversion of DA to 6-OHDA on protein oxidation, DA was incubated with the Fenton system. As seen in chapter 2, this would have allowed some of the DA to be converted to 6-OHDA.

With regard to the effect of DA on the oxidant status of proteins, it has been shown that the incubation of DA with proteins results in an increase in protein carbonyl content. The protein oxidation caused by DA could be related to free radical production during its

auto-oxidation or covalent binding of DA-Q to the protein and subsequent oxidation. DA auto-oxidation might facilitate the generation of HOO• by promoting $O_2^{\bullet-}$ production (Hermida-Ameijeiras *et al*., 2004). $O_2^{\bullet-}$ exists in equilibrium with the HOO• radical (Reiter, 1998). As mentioned previously, HOO• can initiate the protein oxidation process because it is much more lipid soluble and is a much more powerful oxidizing agent than is $O_2^{\bullet-}$ (Reiter, 1998). The addition of the Fenton system to the incubation in the absence of DA also results in a significant increase in carbonyl content. This is most likely caused by •OH formation via the Fenton reaction. In the absence of DA, Fe^{2+} is also the ion directly involved in HOO• production. The increase in the carbonyl content exhibited by the combined action of DA and Fe(II)-EDTA/H_2O_2 may be a consequence of hydroxylation of DA by •OH to yield 6-OHDA. As described in chapter 5, the addition of DA to the Fenton system results in a significant increase in •OH production. This could also be an explanation for the increase in protein oxidation observed in incubations containing DA and the Fenton system.

The results in figure 8.3 show that 6-OHDA is more effective than DA at causing protein oxidation. The reason for this could be related to the fact that 6-OHDA is, as expected on the basis of the additional electron density afforded by the extra hydroxyl substituent, more readily oxidized to 6-hydroxydopamine quinone (6-OHDA-Q) than DA is to DA-Q (Li and Christensen, 1994). Since 6-OHDA oxidizes at a rate much greater than that of DA, it can also generate a substantial amount of H_2O_2, $O_2^{\bullet-}$ and •OH. 6-OHDA mediated ROS formation may also be enhanced in the presence of iron as a result of the reduction of Fe^{3+} by 6-OHDA and 6-OHDA-$SQ^{\bullet-}$ to the Fe^{2+} valence state required for the Fenton reaction. In this way iron also catalyses the oxidation of 6-OHDA (Méndez-Álvarez., 2001). DA mediated ROS generation could also be increased in the presence of iron in the same way, however the reduction of Fe^{3+} to Fe^{2+} will probably occur at a slower rate since DA is a less powerful reducing agent than is 6-OHDA.

The effect of the combined action of 6-OHDA and the Fenton system on protein oxidation does not differ from that found with 6-OHDA alone. A possible reason for this could be a limit to the number of cysteinyl residues and other nucleophilic groups

available to form covalent adducts with 6-OHDA-Q. This hypothesis is based on the assumption that protein oxidation is mediated by the quinone oxidation products of 6-OHDA rather than oxygen free radicals. This hypothesis will be tested further in the next experiment.

8.3 EFFECT OF SELEGILINE ON DOPAMINE AND 6-HYDROXYDOPAMINE INDUCED PROTEIN OXIDATION IN RAT BRAIN HOMOGENATE *IN VITRO*

8.3.1 INTRODUCTION

The previous experiment showed that both DA and 6-OHDA can enhance protein oxidation provoked by the Fenton system. This protein damage could be caused by enhanced production of •OH induced by the addition of DA and 6-OHDA to the Fenton system or could be due to quinone oxidation products of DA and 6-OHDA that bind covalently to nucleophilic groups, such as thiol groups of proteins (Graham *et al*., 1978).

In this experiment SEL was used in the *in vitro* incubation system to inhibit the production of ROS formed during the auto-oxidation and monoamine oxidase metabolism of DA and 6-OHDA. The use of SEL as a radical scavenger should help to establish whether the toxic oxygen species or the quinone oxidation products are responsible for the protein oxidation.

8.3.2 MATERIALS AND METHODS

8.3.2.1 Chemicals and reagents

SEL hydrochloride was purchased from Sigma Chemical Corporation, St Louis, MO, U.S.A. All other chemicals and reagents were purchased as described in section 8.2.2.1.

8.3.2.2 Animals

Animals were housed and maintained as described in appendix one.

8.3.2.3 Brain removal

As described in section 8.2.2.3

8.3.2.4 Homogenate preparation

As described in section 8.2.2.4

8.3.2.5 Protein carbonyl measurement

The assay was carried out as described in section 8.2.2.5 except that in some cases various concentrations of SEL (0.25, 0.5, 0.75 and 1 mM) were added to the incubation before the addition of the DA or 6-OHDA.

8.3.2.6 Statistical analysis

Results were analysed as described in section 2.3.2.5.

8.3.3 RESULTS

As seen in figure 8.4, SEL (1 mM) was ineffective in preventing the protein oxidation induced by either DA alone or the combined action of DA and the Fenton system. SEL (1 mM) on its own had no effect on protein oxidation.

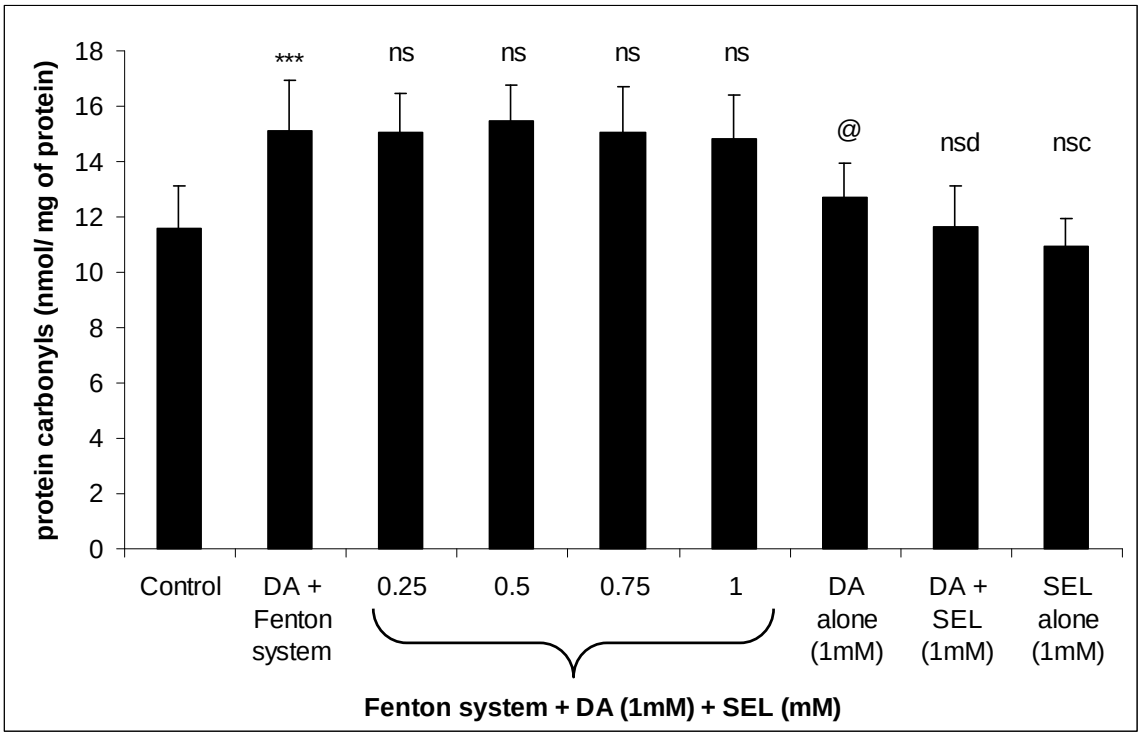


Figure 8.4: Effect of SEL on protein oxidation induced by DA in the presence and absence of the Fenton system. Each bar represents the mean ± SD (n = 5). nsc ($p > 0.05$), @ ($p < 0.05$) and *** ($p < 0.001$) compared to control values; ns ($p > 0.05$) compared to DA + Fenton system values; nsd ($p > 0.05$) compared to DA alone.

Similarly, figure 8.5 shows that SEL has no effect on the protein oxidation induced by either 6-OHDA alone or by the combined action of 6-OHDA and the Fenton system.

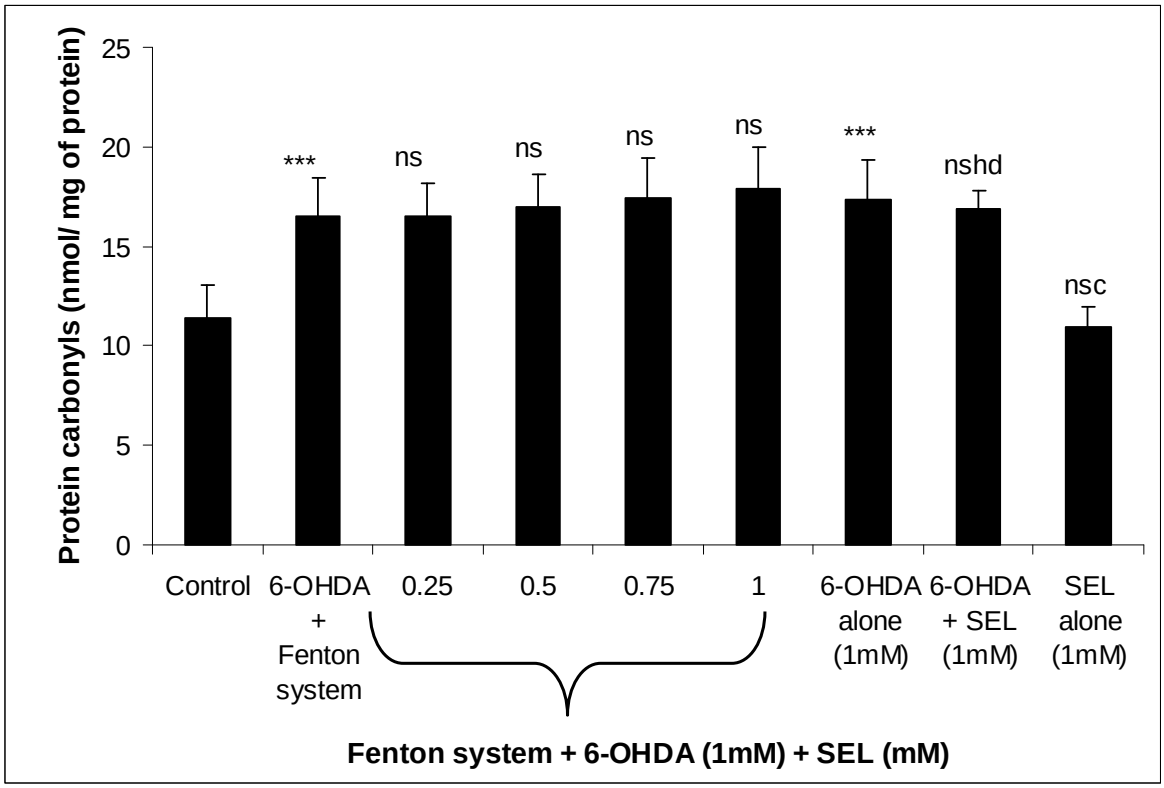


Figure 8.5: Effect of SEL on protein oxidation induced by 6-OHDA in the presence and absence of the Fenton system. Each bar represents the mean ± SD (n = 5). nsc ($p > 0.05$) and *** ($p < 0.001$) compared to control values; ns ($p > 0.05$) compared to 6-OHDA + Fenton system values, nshd ($p > 0.05$) compared to 6-OHDA alone.

8.3.4 DISCUSSION

The results of this study show that the addition of SEL to the incubations was ineffective at inhibiting the increase in protein carbonyl content induced by DA and 6-OHDA alone or in combination with the Fenton system.

Carbonyl groups can be introduced into proteins by reaction with products of lipid peroxidation. Aldehydes such as HNE are formed as by-products of lipid peroxidation. HNE can form adducts with important proteins rendering them inactive (Bharath *et al*., 2002) However, the previous chapter shows that iron stimulated lipid peroxidation is strongly inhibited by DA and 6-OHDA under similar experimental conditions and it is therefore highly unlikely that DA and 6-OHDA stimulate protein oxidation via this mechanism. Chapter 5 showed that DA significantly increases •OH formation by the Fenton system *in vitro* and similar results were also reported for 6-OHDA. Oxidative reactions of proteins are mediated mainly, but not only by •OH. Chapter 2 and chapter 5 show that SEL can scavenge •OH effectively at the high concentrations used in this study and SEL should therefore reduce protein oxidation induced by •OH. The fact that SEL was unable to inhibit the protein oxidation, strongly suggests that •OH produced by the metabolism and auto-oxidation of DA and 6-OHDA are not involved in causing the protein oxidation observed under the present experimental conditions. The most likely explanation for the increased carbonyl content induced by DA and 6-OHDA is covalent addition of DA-Q and 6-OHDA-Q to nucleophilic groups present in the rat brain proteins rather than as the result of oxygen free radicals or any associated lipid peroxidation. Another possibility is that the semiquinone radicals (SQ•) formed during the oxidation of DA and 6-OHDA, rather than •OH may initiate the process of protein oxidation by direct hydrogen abstraction from protein sites.

A possible consequence of the protein oxidation by DA and 6-OHDA is the inhibition of thiol-dependent enzymes such as GSH. The following chapter will investigate whether DA and 6-OHDA can deplete GSH levels *in vitro.* This effect will then be compared to the effect of intraperitoneal LD administration on GSH levels.

8.4 EFFECT OF L-DOPA AND SELEGILINE TREATMENT ON IRON (II)-INDUCED PROTEIN OXIDATION IN THE RAT STRIATUM *IN VIVO*

8.4.1 INTRODUCTION

Oxidative modifications of proteins attract a great deal of attention in age-related neurodegenerative disorders (Aksenov *et al*., 2001). Evidence for enhanced oxidative stress in PD includes findings of oxidative damage to proteins (Alam *et al*., 1997) in PD SNpc along with an increase in iron levels (Dexter *et al*., 1991) and a decrease in GSH levels (Riederer *et al*., 1989; Sofic *et al*., 1992; Sian *et al*., 1994). The previous chapter demonstrates that both DA and 6-OHDA enhance protein oxidation. Since LD treatment increases striatal DA levels, there has been concern that continued use of LD might contribute to neuronal degeneration in PD.

The main aim of this study is to investigate whether acute LD treatment produces an increase in protein oxidation, characterized by an increase in the carbonyl content of protein, in the striatum of male Wistar rats. Striatal iron levels were elevated prior to the commencement of LD treatment by giving the rats an intrastriatal injection of ferrous sulfate (10 nmol).

8.4.2 MATERIALS AND METHODS

8.4.2.1 Chemicals and reagents

L-3,4-dihydroxyphenylalanine (LD), benserazide hydrochloride and SEL hydrochloride were purchased from Sigma Chemical Corporation, St Louis, MO, U.S.A. All other chemicals and reagents were purchased as described in section 8.2.2.1.

8.4.2.2 Animals

Animals were housed and maintained as described in appendix one.

8.4.2.3 Drug treatment

Animals were injected intrastriatally with 2 μl of ferrous sulfate (5 mM) and dosed with LD and SEL for 7 days as described in section 2.5.2.3.2. Control rats received an intrastriatal injection of physiological saline. On the morning after the last dose, rats were sacrificed by cervical dislocation followed by decapitation. Brains were rapidly removed and the iron infused striata of each rat were dissected free and analysed for protein carbonyl content.

8.4.2.4 Homogenate preparation

Each striatum was weighed and homogenized (5 % m/v) in ice cold 0.1 M PBS, pH 7.4. This is necessary to prevent lysosomal damage to the tissue. The homogenate was then used immediately for the assay.

8.4.2.5 Protein carbonyl measurement

The protein carbonyl content was assessed spectrophotometrically according to a modified method of Levine *et al*. (1990). The procedure in section 8.2.2.5 was followed except that the supernatant collected after the addition of the streptomycin sulfate was diluted to 1 mg of protein/ml instead of 2.5 mg of protein/ml. The incubation step at 37 ºC for 1 hour was then excluded and TCA was added to the supernatant (1 mg of protein/ml) to precipitate proteins. The procedure described in section 8.2.2.5 was then followed and results are expressed as protein carbonyl content (nmol/mg of protein).

8.4.2.6 Statistical analysis

Results were analysed as described in section 2.3.2.5.

8.4.3 RESULTS

Figure 8.6 shows that the intrastriatal injection of ferrous sulfate (10 nmol) results in a significant increase in protein oxidation ($p < 0.001$) in rat striatum. Treatment of the rats with LD and SEL alone or in combination has no significant effect on the iron-induced protein oxidation.

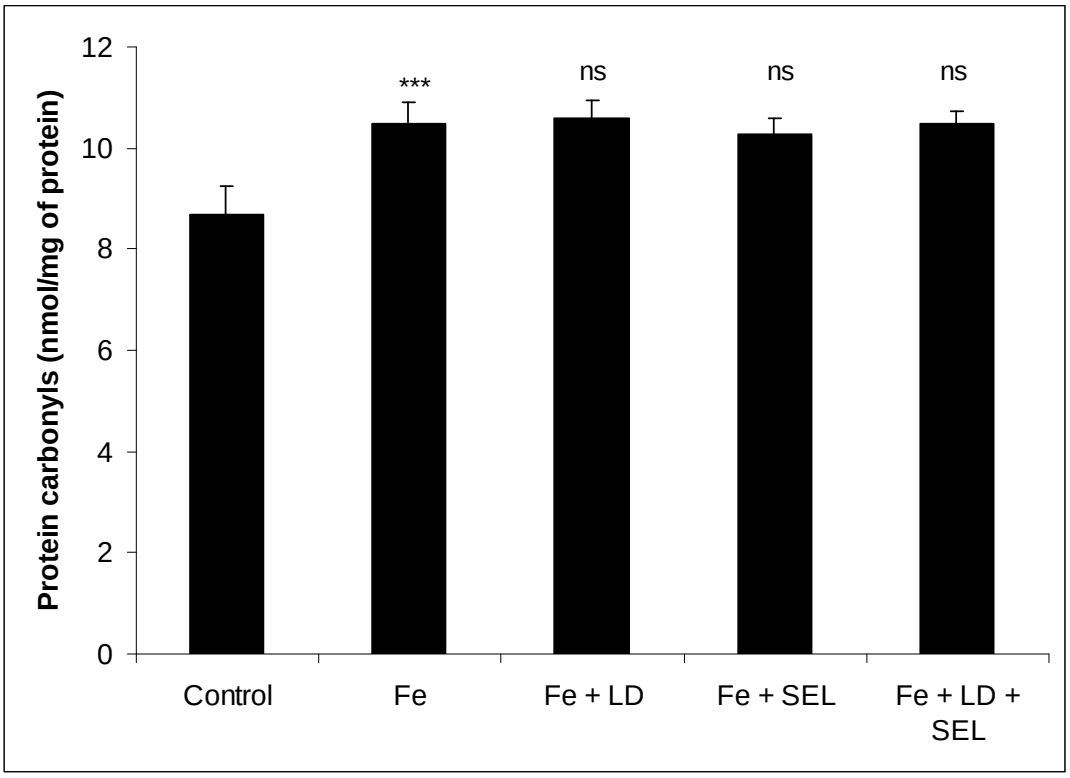


Figure 8.6: Effect of intrastriatal injections of ferrous sulfate followed by LD and SEL treatment on rat striatal protein carbonyl content. Each bar represents the mean ± SD ($n = 5$). *** ($p < 0.001$) compared to the control; ns ($p > 0.05$) compared to rats that received Fe only.

8.4.4 DISCUSSION

The results of this study demonstrate that an increase in the concentration of free ferrous iron in the striatum can result in a significant increase in protein carbonyl content. This increase in protein carbonyl content was not inhibited by treatment with LD, SEL or a combination of these drugs.

The dose of LD used in this study was expected to result in a significant increase in DA levels in the striatum. However, the results of this study do not support the hypothesis that this increase in DA concentration produces oxidative damage to proteins in iron infused striatal rat tissue since there was no significant increase in protein carbonyl content after LD or SEL treatment. A number of factors need to be considered in order to bring together the DA induced oxidative damage to rat brain proteins *in vitro* with LD's apparent lack of neurotoxicity *in vivo.* One important consideration is that the magnitude and duration of the increase in DA levels may not have been sufficient to produce a significant increase in protein carbonyl content. For example, the dose of LD (10 mg/kg/bd) used in this study was expected to produce a two-fold increase in striatal DA content, as seen in chapter 4, however the administration of 10 – 15 mg/kg of methamphetamine increases extracellular DA content 20- to 80-fold (Stephans and Yamamoto, 1994).

It is also possible that the reported ability of LD to increase GSH levels in dopaminergic neurons (Mytilineou *et al*., 1993; Han *et al*., 1996) may protect striatal proteins from damage. However, this reported increase in GSH induced by LD also contradicts the fact that the quinone oxidation products of LD, DA and 6-OHDA bind covalently to thiol groups (SH groups) which should theoretically deplete GSH levels. The following chapter will investigate the effect of DA and 6-OHDA on GSH levels *in vitro* and the effect of LD administration on striatal GSH levels *in vivo*.

CHAPTER NINE

TOTAL GLUTATHIONE CONTENT

9.1 INTRODUCTION

The tripeptide glutathione (GSH) is the most abundant nonprotein intracellular thiol (-SH) compound in mammalian cells, (Bharath *et al*., 2002) and is present in concentrations up to 12 mM (Dringen, 2000). GSH is synthesized from its constituent amino acids (glutamate, cysteine and glycine) in two consecutive steps catalyzed by γ-glutamyl cysteine synthase (GCS) and glutathione synthase. GCS uses glutamate and cysteine as substrates forming γ-glutamylcysteine which is then combined with glycine in a reaction catalyzed by glutathione synthase to form GSH (see figure 9.1).

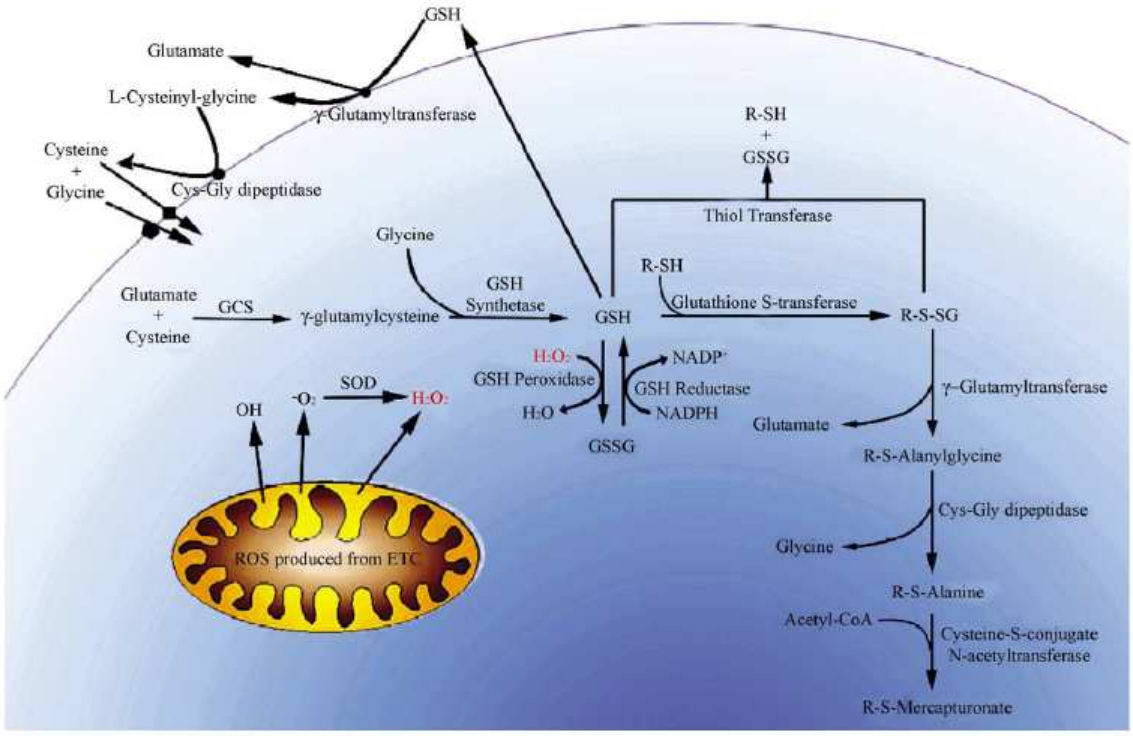


Figure 9.1: A schematic representation of the synthesis and metabolism of GSH (Bharath *et al*., 2002)

Glutathione plays a crucial role in the primary cellular defense against oxidative stress by reacting both nonenzymatically with ROS ($O_2^{\bullet-}$, •OH and NO•) (Saez *et al*., 1990; Winterbourne and Metodiewa, 1994; Singh *et al*., 1996) and also acting as an electron donor in the reduction of H_2O_2 catalyzed by glutathione peroxidase (GPx) (Chance *et al*., 1979). GSH may also protect neurons from the accumulation of protein aggregates which form Lewy bodies within the cell (Shimura *et al*., 2001). HNE is an aldehyde formed as a by-product during lipid peroxidation. It has been proposed that HNE can integrate into membranes affecting *in vivo* membrane fluidity (Chen and Yu, 1994) and can also form adducts with important biological proteins. It has been found that GSH conjugates with HNE and therefore prevents it from incorporating into membranes (Chen and Yu, 1994; Chen and Yu, 1996) and from forming conjugates with proteins (Subramanian *et al*., 1997). Another important function of GSH is that it protects proteins from oxidation by conjugating with thiol groups to form protein-SS-G mixed disulfides which can be re-reduced to protein and GSH by glutathione reductase (GR), thioredoxin or protein disulfide isomerase (Ravindranath and Reed, 1990). Figure 9.2 summarizes some of the oxidation reactions inhibited by GSH.

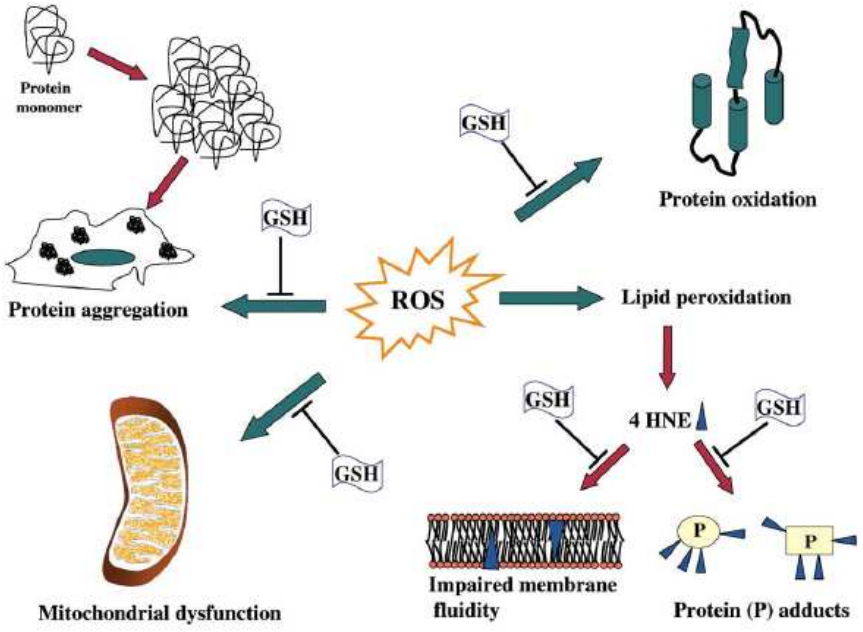


Figure 9.2: GSH has the ability to inhibit oxidative damage to a number of important biomolecules (Bharath *et al*., 2002).

In dopaminergic neurons *in vivo,* GSH can bind to quinones formed during the oxidation of DA and 6-OHDA and prevent these quinones from reacting with protein thiol groups (Fornstedt *et al*., 1990a; Hastings *et al*., 1996). However, the binding of these quinones to GSH may deplete GSH stocks and therefore impair GSH dependent detoxification. Several studies have shown that the relative variations in GSH levels in different brain regions are cortex > cerebellum > hippocampus > striatum > SN (Abbott *et al*, 1990; Chen *et al*., 1989; Kang *et al*., 1999) There is therefore an inverse relationship between levels of DA and levels of GSH in the brain.

In PD, there is a further reduction in GSH levels within the SN. The depletion of GSH levels in Parkinsonian brains cannot be explained by increased oxidation of GSH to GSSG as levels of both are found to be decreased. The activity of GCS in the SN of PD patients is normal and the decrease in GSH can therefore also not be explained by a decrease in GSH synthesis (Sian *et al*., 1994). The decrease in GSH observed in PD may be caused by the increased synthesis and turnover of DA in surviving dopaminerigic neurons in the SN and the striatum. This chapter therefore investigates the effect of DA, 6-OHDA and SEL on GSH levels *in vitro* and the effect of LD and SEL administration on striatal GSH levels *in vivo.*

The spectrophotometric procedures used to assay for GSH in this chapter are based on the method of Ellman (Ellman, 1958; Ellman, 1959), who reported that 5,5-dithiobis-(2-nitrobenzoic acid) is reduced by thiol groups (SH) to form 1 mole of 2-nitro-5-mercaptobenzoic acid per mole of SH. The nitromercaptobenzoic acid anion has an intense yellow color that can be used to measure SH groups.

9.2 EFFECT OF DOPAMINE AND 6-HYDROXYDOPAMINE ON THE IRON (II)-INDUCED LOSS OF TOTAL GLUTATHIONE IN RAT BRAIN HOMOGENATE *IN VITRO*

9.2.1 INTRODUCTION

The degeneration of dopaminergic neurons in PD is attributed in large measure to strong oxidative stress within the degenerating *substantia nigra* (SNpc). The two main biochemical phenomena leading to increased ROS in the parkinsonian SNpc are an increase in iron levels (Dexter *et al*., 1989a) and a reduced antioxidant defense level (Riederer *et al*., 1989; Sofic *et al*., 1992; Sian *et al*., 1994).

PD is associated with a decrease in GSH levels (Sofic *et al*., 1992). This decrease in GSH observed in PD may affect the ability of dopaminergic neurons to protect themselves against lipid peroxidation, protein oxidation and mitochondrial dysfunction (figure 9.2). The decrease in GSH may, in part, be due to the covalent binding of quinones formed during the auto-oxidation of DA and 6-OHDA to the GSH molecule resulting in the impairment of GSH-dependent detoxification and enhancing the formation of ROS such as H_2O_2 and •OH. The enhanced production of ROS during the metabolism and auto-oxidation of DA and 6-OHDA may also contribute to a loss of GSH stocks.

This study investigates the effect of the metabolism and auto-oxidation of DA and 6-OHDA on total glutathione content in rat brain homogenate. In addition, the study also investigates the effects provoked by the presence of the Fenton system on the depletion of GSH caused by DA and 6-OHDA.

9.2.2 MATERIALS AND METHODS

9.2.2.1 Chemicals and reagents

DA hydrochloride, 6-OHDA hydrobromide and 5,5-dithiobis-(2-nitrobenzoic acid) (DTNB) were purchased from Sigma Chemical Corporation, St Louis, MO, U.S.A. All other reagents used were of the highest quality available.

Stock solutions of DA and 6-OHDA were prepared in 1 mM KCl (pH 2.0) to prevent their immediate auto-oxidation. Fresh stock solutions of Fe^{2+} were prepared in deaerated water immediately before each experiment to prevent oxidation of the Fe^{2+} to Fe^{3+}.

9.2.2.2 Preparation of the standard curve

Reduced glutathione was used to prepare the standard curve. An aliquot of 0.5 ml of varying concentrations of GSH (2-10 µM dissolved in PBS, pH 7.4) was added to test tubes and mixed with 1.5 ml of 0.2 M Tris buffer, pH 8.2 and 0.1 ml of 0.01 M DTNB. The mixture was then brought to 10 ml with 7.9 ml of absolute methanol. The absorbance was read at 412 nm using a Shimadzu UV-160A UV-visible recording spectrophotometer and a calibration curve was constructed (appendix six).

9.2.2.3 Animals

Animals were housed and maintained as described in appendix one.

9.2.2.4 Brain removal

Rats were sacrificed by cervical dislocation, followed by decapitation, and the brains were rapidly removed for use in experiments as described in appendix two. The brains were either used immediately or stored at -70 ºC until needed.

9.2.2.5 Homogenate preparation

Each brain was weighed and homogenized (20 % m/v) in ice cold 0.1 M PBS, pH 7.4 in a glass teflon homogenizer. The homogenate was then used immediately for the assay.

9.2.2.6 Measurement of total glutathione content

The total GSH content was assessed spectrophotometrically according to a modified method of Sedlak and Lindsay (1968).

Briefly, 0.5 ml of rat brain homogenate (20 % m/v) was incubated at 37 ºC for 5 minutes to reach the working temperature. Hydrogen peroxide (100 µM), ascorbate (100 µM), EDTA (240 µM) and ferrous sulfate (200 µM) were incorporated into the incubation and the mixture incubated for 60 minutes. To investigate the effect of DA and 6-OHDA on GSH content, various concentrations of DA or 6-OHDA (0.25, 0.5, 0.75 and 1 mM) were added to the incubation before the addition of the ferrous sulfate (the final volume of the incubation mixture was 1 ml). Immediately after the incubation, 0.5 ml of the incubation mixture was added to test tubes containing 1.5 ml of Tris buffer (pH 8.2), 0.1 ml of 0.01 M DTNB and 7.9 ml of absolute methanol. The resultant mixture was then allowed to stand at room temperature, with occasional shaking, for 30 minutes. Thereafter, the mixture was centrifuged at 3000 x g for 15 minutes and the absorbance of the supernatant was read at 412 nm. The GSH levels were determined from a standard curve generated from GSH and are expressed as total GSH (nmol/mg of tissue).

9.2.2.7 Statistical analysis

Results were analysed as described in section 2.3.2.5.

9.2.3 RESULTS

Figure 9.3 shows how the incubation of rat brain homogenate with DA or 6-OHDA at 37 ºC for 60 minutes causes a significant ($p < 0.001$) decrease in GSH content (± 19% reduction in total GSH for incubations containing DA (1 mM) and a ± 58% reduction in total GSH for incubations containing 6-OHDA (1 mM) compared to the control values). The addition of Fenton reagents to the incubation of rat brain homogenate also causes a significant decrease in the GSH content of rat brain homogenate (± 12% reduction in GSH levels compared to the control). However, the combined action of DA or 6-OHDA with the Fenton system significantly augments the decrease in GSH obtained with the use of DA or 6-OHDA alone. In addition to this, the decrease in GSH levels observed in incubations containing both the Fenton system and either DA or 6-OHDA is dependent on the concentration of DA or 6-OHDA used. Interestingly, the augmentation observed with the combined action of DA + Fenton system which caused a further ± 60% decrease in GSH content compared to DA alone was greater than that observed with the combined action of 6-OHDA + Fenton system which caused a further ± 28% decrease in GSH compared to 6-OHDA alone.

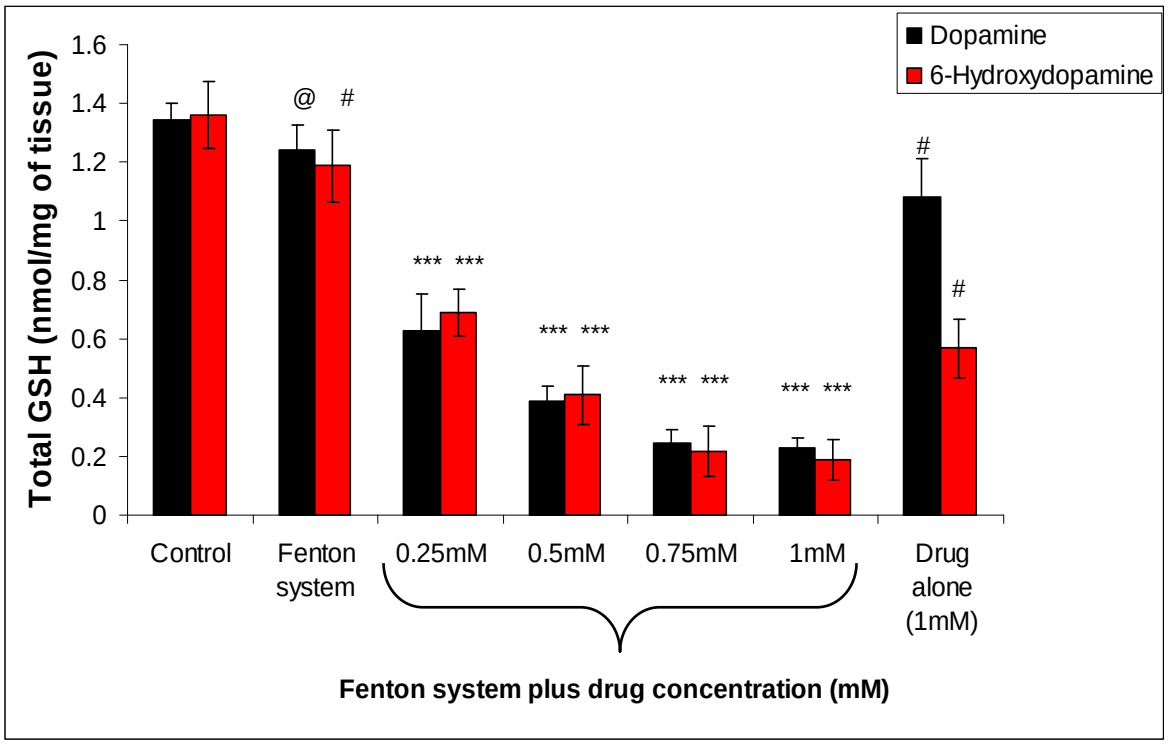


Figure 9.3: Effect of DA and 6-OHDA on total GSH content in the presence and absence of the Fenton system. Each bar represents the mean ± SD (n = 5). @ ($p < 0.01$) and # ($p < 0.001$) compared to control values; *** ($p < 0.001$) compared to the Fenton system group.

9.2.4 DISCUSSION

The present study shows that the incubation of rat brain homogenate with DA or 6-OHDA results in a marked decrease in total GSH. The reduction in GSH content was greater for 6-OHDA (± 58 % reduction) than for DA (± 19 % reduction) which can be attributed to the higher rate of autoxidation of 6-OHDA. In addition, the presence of the Fenton system also causes a significant fall in total GSH content of rat brain homogenate. The effect observed with the combined action of Fenton system + DA caused a greater reduction in GSH than DA alone and this can be attributed to the capacity of iron to catalyze the auto-oxidation of DA as well as the ability of •OH, formed during the Fenton

reaction, to convert DA to 6-OHDA. The combined effect of Fenton system + 6-OHDA also resulted in a greater loss of total GSH than 6-OHDA alone.

The effects of 6-OHDA and DA both in the presence and absence of the Fenton system on total GSH reported above could be related to the generation of H_2O_2 and •OH formed during the auto-oxidation and MAO mediated metabolism of these compounds. However, the inability of catalase (Chakrabarti *et al*., 1990) and •OH scavengers (Chakraborty *et al*., 2001) to inhibit the reduction of protein thiol (SH) content induced by DA under different experimental conditions weakens this hypothesis. Another possible explanation for the decrease in GSH caused by DA and 6-OHDA is the oxidation of these compounds to DA-Q and 6-OHDA-Q and the subsequent reaction of these quinones with the thiol groups on the GSH molecule. Evidence that these quinones can react with SH groups in the brain is the finding that cysteinyl adducts, such as 5-cysteinyl-dopamine and quinone adducts, have been encountered in rat, guinea pig and human brain (Carlsson and Fornstedt, 1991; Fornstedt *et al*., 1990b).

Chapter 7 shows that DA and 6-OHDA are able to inhibit hydroxyl radical induced lipid peroxidation. However, one of the possible consequences of GSH depletion is that it may eventually stimulate lipid peroxidation, indirectly, by weakening the endogenous antioxidant defenses against lipid peroxidation i.e. the GSH-dependent detoxification of H_2O_2 and ROS (Boots *et al*., 2002). A decrease in GSH levels could also cause an increase in the protein oxidation (chapter 8) and mitochondrial dysfunction (chapter 5) induced by DA and 6-OHDA since GSH prevents the quinone oxidation products of these compounds from conjugating with protein SH groups (Hastings *et al*., 1996).

The following experiment investigates whether SEL, a selective MAO-B inhibitor and a scavenger of •OH, can protect against the loss of total GSH induced by DA and 6-OHDA.

9.3 EFFECT OF SELEGILINE ON DOPAMINE AND 6-HYDROXYDOPAMINE INDUCED LOSS OF TOTAL GLUTATHIONE IN RAT BRAIN HOMOGENATE *IN VITRO*

9.3.1 INTRODUCTION

The previous experiment shows that the incubation of rat brain homogenate with DA or 6-OHDA, both in the absence and in the presence of the Fenton system results in a significant reduction in total GSH. This decrease in GSH content could be attributed to an increased production of H_2O_2 and •OH generated by the addition of DA and 6-OHDA to the Fenton system. It could also be brought about by the covalent binding of DA-Q and 6-OHDA-Q to the GSH molecule.

There are two important mechanisms that prevent DA oxidation. The first mechanism is the incorporation of DA into monoamine transporter vesicles (VAMT). The low pH inside these vesicles prevents the oxidation of DA (Arriagada *et al*., 2004). However, this is not relevant when working with homogenate since the integrity of the cells has been damaged. The second mechanism inhibiting the oxidation of DA is the breakdown of DA by MAO (Weingarten and Zhou, 2001; Arriagada *et al*., 2004). SEL, a MAO-B inhibitor should therefore favour DA oxidation and the formation of quinone oxidation products. On the other hand, SEL inhibits the formation of H_2O_2 formed from the breakdown of DA and also scavenges •OH (chapter 6). The use of SEL in the incubations should therefore help to establish whether the ROS or the quinone oxidation products are responsible for the loss of GSH induced by 6-OHDA and DA.

9.3.2 MATERIALS AND METHODS

9.3.2.1 Chemicals and reagents

SEL hydrochloride was purchased from Sigma Chemical Corporation, St Louis, MO, U.S.A. All other chemicals and reagents were purchased as described in section 9.2.2.1.

9.3.2.2 Preparation of the standard curve

As described in section 9.2.2.2

9.3.2.3 Animals

Animals were housed and maintained as described in appendix one.

9.3.2.4 Brain removal

As described in section 9.2.2.4

9.3.2.5 Homogenate preparation

As described in section 9.2.2.5

9.3.2.6 Measurement of total glutathione content

The total GSH content was assessed spectrophotometrically according to a modified method of Sedlak and Lindsay (1968).

The assay was carried out as described in section 9.2.2.6 except that in some cases various concentrations of SEL (0.25, 0.5, 0.75 and 1 mM) were added to the incubation before the addition of the DA or 6-OHDA.

9.3.2.7 Statistical analysis

Results were analysed as described in section 2.3.2.5.

9.3.3 RESULTS

As illustrated in figure 9.4, the addition of SEL (0.25, 0.5, 0.75 and 1 mM) to incubations is ineffective at inhibiting the loss of GSH induced by the combined action of DA and the Fenton system. However, SEL did cause an increase in GSH content in incubations containing DA and SEL when compared with the value obtained in incubations containing only DA (± 8 % increase in total GSH).

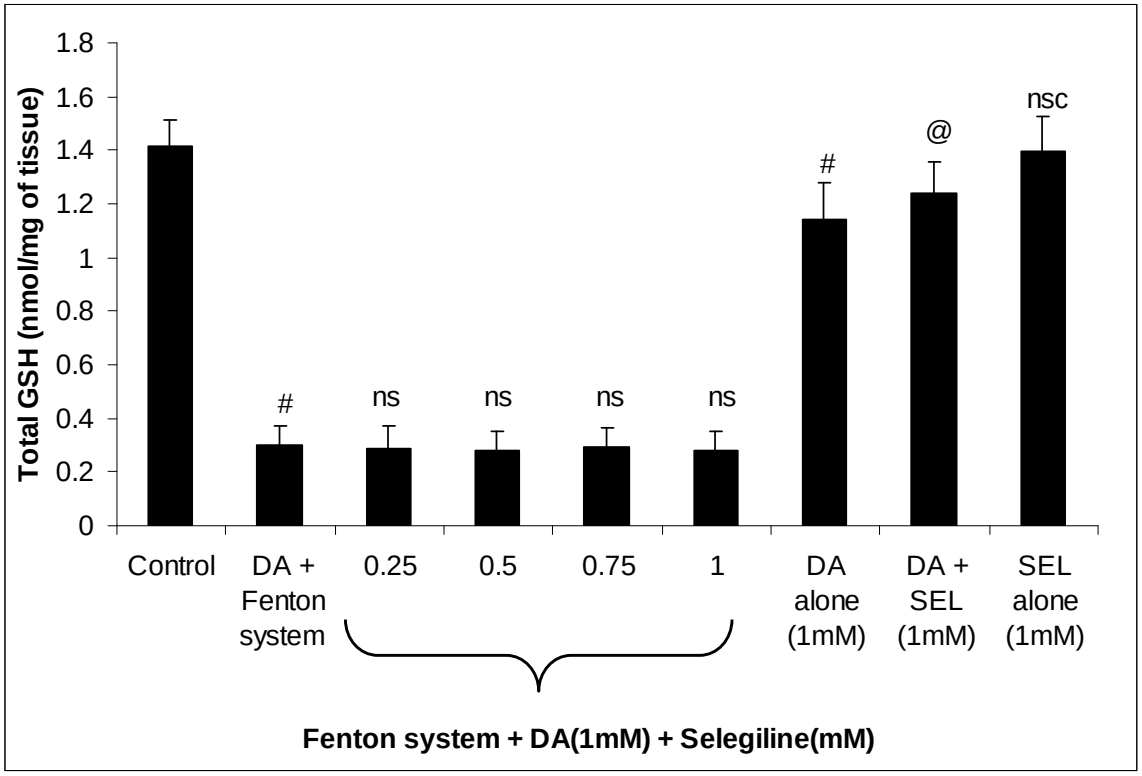


Figure 9.4: Effect of SEL on the loss of total GSH induced by DA in the presence and absence of the Fenton system. Each bar represents the mean ± SD (n = 5). nsc ($p > 0.05$) and # ($p < 0.001$) compared to control values; ns ($p > 0.05$) compared to DA + Fenton system values; @ ($p < 0.01$) compared to DA alone.

Total Glutathione Content

Figure 9.5 shows that SEL has no effect on the loss of GSH induced by either 6-OHDA alone or the combined action of 6-OHDA with the Fenton system.

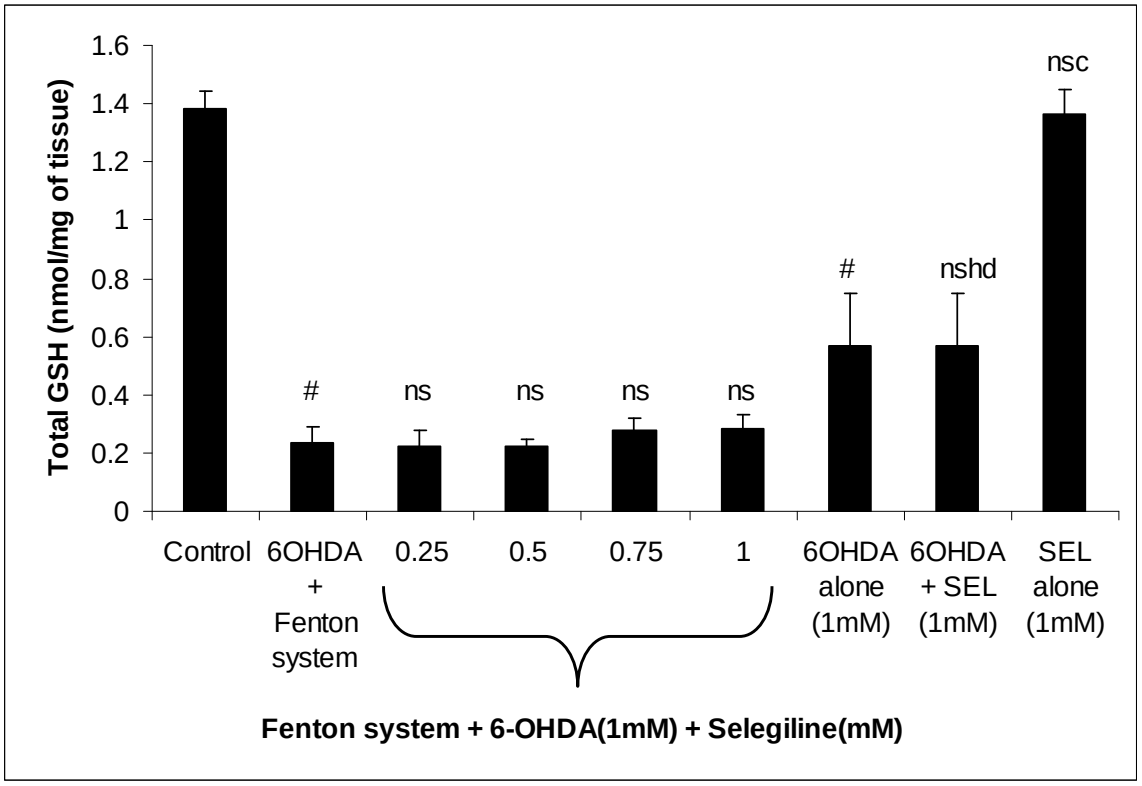


Figure 9.5: Effect of SEL on the loss of total GSH induced by 6-OHDA in the presence and absence of the Fenton system. Each bar represents the mean ± SD (n = 5). Nsc ($p > 0.05$) and # ($p < 0.001$) compared to control values; ns ($p > 0.05$) compared to 6-OHDA + Fenton system values, nshd ($p > 0.05$) compared to 6-OHDA alone.

9.3.4 DISCUSSION

The results of this study show that SEL is unable to inhibit the reduction of total GSH induced by the combined action of (DA + Fenton system) or (6-OHDA + Fenton system). The results also show that SEL is unable to inhibit the reduction in GSH induced by 6-OHDA in the absence of the Fenton system.

SEL has the capacity to reduce the production of H_2O_2 as well as the oxygen radicals most likely to be involved in the oxidation of thiol groups (•OH and HOO•). Chapters 5 and 6 show that SEL, at the concentrations used in this study, reduces the production of •OH, $O_2^{\bullet -}$ and consequently HOO•. The most likely explanation for the inability of SEL to inhibit the loss of GSH induced by 6-OHDA, (DA + Fenton system) and (6-OHDA + Fenton system) is that under these circumstances, the loss of GSH is caused predominantly by the quinone oxidation products of DA and 6-OHDA rather than ROS.

The results of the present study also show that SEL is able to inhibit the loss of GSH induced by the incubation of rat brain homogenate with DA alone. However, the amount of inhibition produced is small considering the large concentration of SEL used (1 mM). This suggests that the generation of ROS during the auto-oxidation and MAO mediated metabolism of DA only plays a minor role in the loss of total GSH induced by this neurotransmitter.

9.4 EFFECT OF L-DOPA AND SELEGILINE TREATMENT ON TOTAL GLUTATHIONE CONTENT IN THE RAT STRIATUM *IN VIVO*

9.4.1 INTRODUCTION

GSH depletion may represent one of the earliest biochemical defects in PD (Blum *et al*., 2001). Although GSH is not the only antioxidant depleted during PD, the magnitude of GSH depletion appears to parallel the severity of the disease and occurs prior to other hallmarks of the disease (Perry and Yong, 1986; Jenner, 1993).

The previous experiments in this chapter have shown that DA and 6-OHDA are capable of reducing total GSH levels in rat brain homogenate. LD administration may also induce oxidative damage by depleting GSH levels (Spencer *et al*., 1995). However, Mytilineou *et al*. (1993) and Han *et al*. (1996) reported that LD had the ability to increase GSH levels in astroglial cultures.

The main aim of this study is to investigate the effect of acute LD and SEL administration on striatal GSH levels.

9.4.2 MATERIALS AND METHODS

9.4.2.1 Chemicals and reagents

L-3,4-dihydroxyphenylalanine (LD), benserazide hydrochloride and SEL hydrochloride were purchased from Sigma Chemical Corporation, St Louis, MO, U.S.A. All other chemicals and reagents were purchased as described in section 9.2.2.1.

9.4.2.2 Animals

Animals were housed and maintained as described in appendix one.

9.4.2.3 Drug treatment

Animals were injected intrastriatally with 2 µl of ferrous sulfate (5 mM) and dosed with LD and SEL for 7 days as described in section 2.5.2.3.2. Control rats received an intrastriatal injection of physiological saline. On the morning after the last dose, rats were sacrificed by cervical dislocation followed by decapitation. Brains were rapidly removed and the iron infused striata of each rat were dissected free and analysed for total GSH content.

9.4.2.4 Homogenate preparation

Each striatum was weighed and homogenized (5 % m/v) in ice cold 0.1 M PBS, pH 7.4. The homogenate was then used immediately for the assay.

9.4.2.5 Measurement of total glutathione content

The total GSH content was assessed spectrophotometrically according to a modified method of Sedlak and Lindsay (1968).

Briefly, to 0.5 ml of homogenate (5 % m/v), 1.5 ml of 0.2 M Tris buffer (pH 8.2), 0.1 ml of 0.01 M DTNB and 7.9 ml of methanol was added and incubated for 30 minutes at room temperature. The incubation mixture was then centrifuged at 3000 x g for 15 minutes and the absorbance of the supernatant read at 412 nm. The GSH levels were determined from a standard curve generated from GSH and are expressed as total GSH (nmol/mg of tissue).

9.4.2.6 Statistical analysis

Results were analysed as described in section 2.3.2.5.

9.4.3 RESULTS

Figure 9.6 shows that the intrastriatal injection of ferrous sulfate (10 nmol) reduced the total GSH content in rat striatum ($p < 0.05$). Treatment of the rats with LD and SEL alone or in combination had no significant effect on the decrease in total GSH induced by the intrastriatal injection of iron.

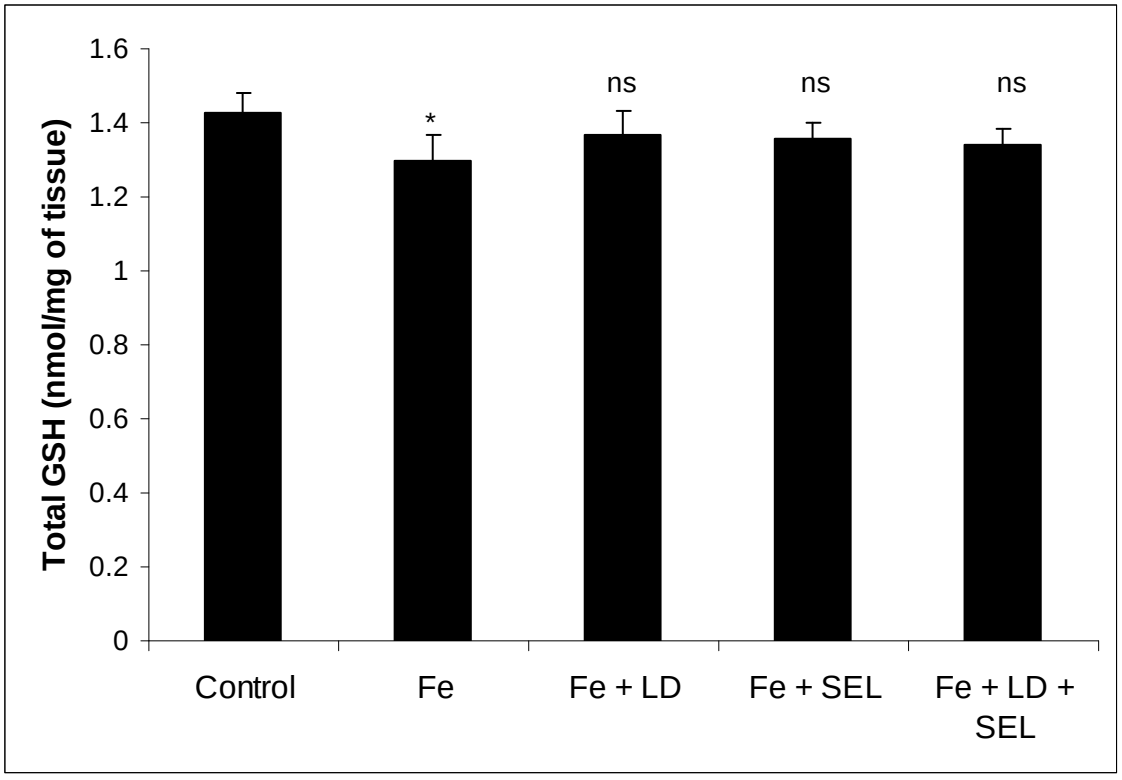


Figure 9.6: Effect of intrastriatal injection of ferrous sulfate followed by LD and SEL treatment on rat striatal GSH content. Each bar represents the mean ± SD (n = 5). * ($p < 0.05$) compared to the control; ns ($p > 0.05$) compared to rats that received Fe only.

9.4.4 DISCUSSION

This study demonstrates that an increase in the concentration of free ferrous ions in the striatum reduces total GSH levels. This decrease in GSH is not attenuated or enhanced by treatment with LD, SEL or a combination of these drugs.

Under normal circumstances, GSH constantly clears H_2O_2, thus preventing the formation of •OH. GSH also conjugates with quinones formed during the oxidation of DA and 6-OHDA and prevents these from forming deleterious adducts with proteins (Bharath *et al*., 2002) and from facilitating the release of iron from ferritin (Andersen, 2001). Under conditions of GSH depletion, as observed in PD this protection is attenuated resulting in oxidative stress. It has been proposed that the release of iron from ferritin alters the homeostasis of mitochondrial calcium leading to the formation of ROS and the depletion of tissue GSH levels (Youdim and Riederer, 1993). The ability of DA and 6-OHDA to release iron (II) from ferritin will be investigated in the following chapter.

Despite the decrease in total GSH levels induced by DA and 6-OHDA observed in the previous experiments, the dosage of LD used in this experiment (10 mg/kg/bd) did not enhance the loss of GSH induced by the intrastriatal injection of iron. Once again the toxicity of DA *in vitro* does not match the lack of toxicity observed with the administration of LD *in vivo*. In addition to this, SEL treatment, either alone or in combination with LD does not result in any significant changes in GSH levels.

CHAPTER TEN

IRON INTERACTION STUDIES

10.1 INTRODUCTION

As an essential component of heme groups and iron sulfur clusters, iron is important for the transport (hemoglobin), storage (myoglobin) and use of oxygen (cytochromes, cytochrome oxidase and iron sulfur proteins) for respiration (Halliwell, 1992; Riemer *et al*., 2004). Iron is also an essential component in the active sites of many enzymes, including catalase (Halliwell, 1992).When iron is present in excess, it has the potential to harm biological systems since in redox active form it catalyses the generation of ROS, including •OH via the Fenton reaction (Crichton *et al*., 2002). An example of this "iron paradox" operates in the dopaminergic neurons of the SN, the same group of cells that are lost in PD. On the one hand, iron is required as an essential cofactor by the enzyme tyrosine hydroxylase for the synthesis of dopamine. On the other hand, iron promotes the oxidation of DA, releasing H_2O_2 in the process. H_2O_2 can then give rise to the highly toxic •OH (Kaur and Andersen, 2002).

Sofic *et al*. (1992) demonstrated that total iron levels in the SN are elevated in PD patients. Intracellular iron levels are stringently regulated as a labile iron pool, which provides optimum iron levels for a multitude of biochemical processes and limits the availability of free iron for the generation of ROS (Bharath *et al*., 2002). Ferritin (figure 10.1) is the major iron storage protein in the brain and maintains iron in a nonreactive form in the cell (Babincová *et al*., 2005). Ferritin is composed of 24 sub-units arranged in 432 symmetry to form a hollow protein shell enclosing a cavity 80 Angstroms in diameter (Jameson *et al*., 2004). Iron incorporation into ferritin involves oxidation of Fe^{2+} to Fe^{3+} and deposition of the Fe^{3+} within the hollow protein shell essentially as ferrihydrite (Crichton *et al*., 2002). Ferritin can store up to 4500 iron atoms per molecule (Crichton *et al*., 2002). It is uncertain whether the excess iron in the brains of PD patients

is in a free form or whether it is bound to ferritin (Riederer *et al*., 1989; Dexter *et al*., 1990; Jellinger *et al*., 1990; Mann *et al*., 1994). Griffiths *et al*. (1999) demonstrated that in PD patients, ferritin is heavily loaded with iron and that even if there is an increase in ferritin to counter excess iron levels, the ferritin molecules are saturated with iron.

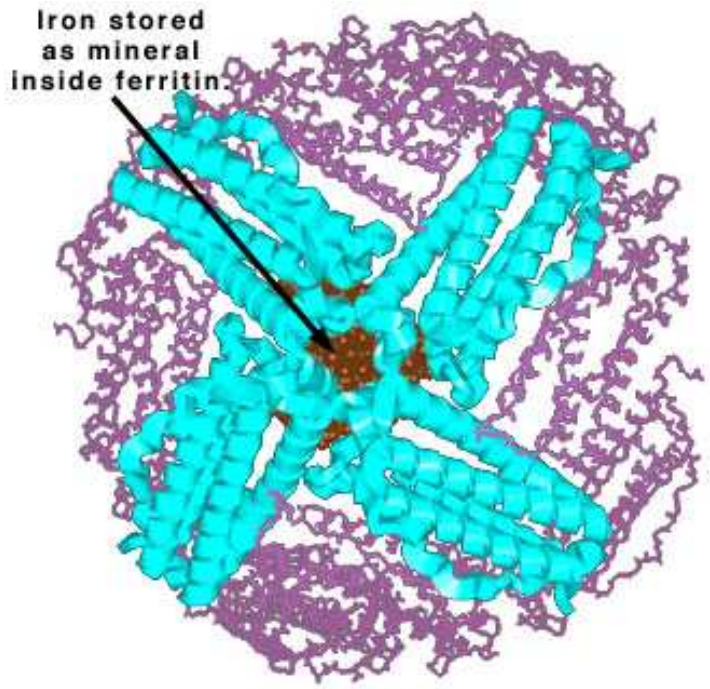


Figure 10.1: A three-dimensional representation showing ferritin, the iron-storage protein in the body. Ferritin has a spherical shape, and iron (brown) is stored as a mineral inside the sphere.
(http://www.chemistry.wustl.edu/~courses/genchem/Tutorials/Ferritin/iron_06.htm).

The superoxide (Yoshida *et al*., 1995) or catechol (Linert *et al*., 1996; Babincová and Babinec, 2005) mediated release of iron from ferritin increases the labile iron pool and also the fraction of iron capable of reacting with H_2O_2 (Double *et al*., 1998). Furthermore, iron promotes the auto-oxidation of DA in dopaminergic neurons, releasing additional H_2O_2 (Ben-Shachar *et al*., 1995). The increased generation of •OH via Fenton chemistry could also increase the conversion of DA to 6-OHDA in the presence of an abnormal accumulation of iron. The neurotoxicity of 6-OHDA has been linked to the release of iron

from ferritin (Montinero and Winterbourne, 1989). Desferrioxamine, an iron chelator, reduces 6-OHDA lesioning of the the nigrostriatal DA system (Ben-Shachar *et al*., 1991). The removal of iron from ferritin is achieved by reducing iron from the Fe^{3+} oxidation state to the Fe^{2+} oxidation state. In the Fe^{2+} state, iron breaks away from the lattice as the Fe^{2+} ion. The positive charge on the Fe^{2+} ion attracts the electronegative oxygen atoms of water and a water "cage" forms around the ion. Thus, iron becomes soluble as a hydrated Fe^{2+} ion and can be released from the ferritin protein via the channels in the protein shell. (http://www.chemistry.wustl.edu/~courses/genchem/Tutorials/Ferritin/iron_06.htm).

Linert *et al*. (1996) proposed a plausible mechanism for the production of cytotoxins in PD (figure 10.2). Fe^{2+} interacts with H_2O_2 via Fenton's reaction producing •OH or ferryl species. These can convert the neurotransmitter DA to the neurotoxin 6-OHDA, which is a powerful reducing agent. The production of 6-OHDA is then followed by the reduction and release of iron, as Fe^{2+} from ferritin. In other words, an autocatalytic cycle is formed which continuously produces cytotoxic species.

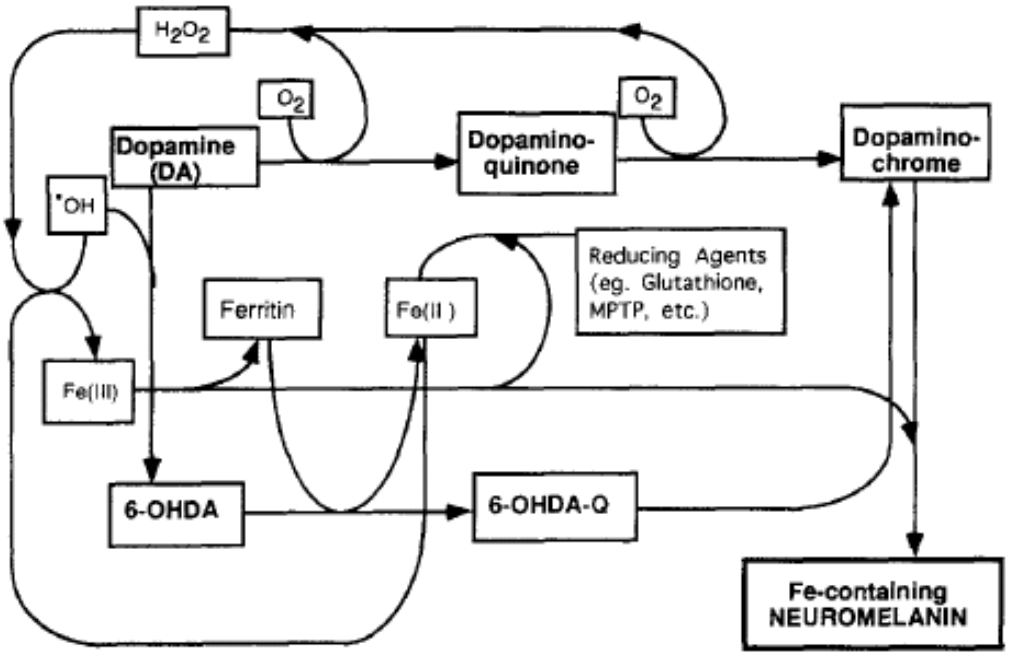


Figure 10.2: The production of 6-OHDA followed by the release of Fe^{2+} from ferritin which may enable sustained production of cytotoxic species (Linert *et al*., 1996).

This chapter aims to determine whether an interaction exists between DA, 6-OHDA, SEL and iron (both in the ferrous and ferric oxidation state). The binding of Fe^{2+} or Fe^{3+} by these agents could prevent iron from catalyzing the formation of ROS by the Fenton reaction. Secondly, the chapter will also investigate the ability of DA and 6-OHDA to release iron as Fe^{2+} from the iron storage protein ferritin. The results of these studies will help to explain the peculiar ability of 6-OHDA and DA to increase iron-induced •OH production (chapter 6) and protein oxidation (chapter 8) on the one hand, but reduce lipid peroxidation (chapter 7) under the same conditions on the other hand.

10.2 EFFECT OF DOPAMINE AND 6-HYDROXYDOPAMINE ON THE RELEASE OF IRON (II) FROM FERRITIN AND THE FORMATION OF A FERROZINE-IRON (II) COMPLEX

10.2.1 INTRODUCTION

The quantitation of iron in biological samples can be achieved using a variety of methods including atomic absorption spectroscopy (Hoepken *et al*., 2004), paramagnetic resonance spectroscopy (Woodmansee and Imlay, 2002), densitometric analysis (LeVine *et al*., 1998), and colorimetric quantitation (Fish, 1988; Gay *et al*., 1999). Ferrozine (figure 10.3) is an effective chelator of ferrous iron (Fe^{2+}) and has been used for the colorimetric determination of iron in biological samples (Ceriotti and Ceriotti, 1980, Riemer *et al*., 2004). Ferrozine binds Fe^{2+} (figure 10.4), but not Fe^{3+}, into a purple complex that absorbs strongly at 562 nm (Babincová and Babinec, 2005). As shown in figure 10.2, ferrozine binds Fe^{2+} with a metal to ligand ratio of 1:3.

After uptake into cells, Fe^{2+} may encounter a ferritin molecule, which incorporates iron as Fe^{2+}, oxidizing it to Fe^{3+}, and traps the Fe^{3+} within the protein shell, alternatively the iron may also be incorporated into other protein complexes (Crichton *et al*., 2002). Such complexed iron is not available for quantitation using colorimetric assays. To quantify the total iron content of a cell (free iron and protein-bound iron) iron must first be released from the proteins (Riemer *et al*., 2004). Therefore, the spectrophotometric measurement of the formation of Fe(II)-ferrozine complexes is an ideal way to measure the release of iron, as Fe^{2+} from ferritin mediated by DA and 6-OHDA.

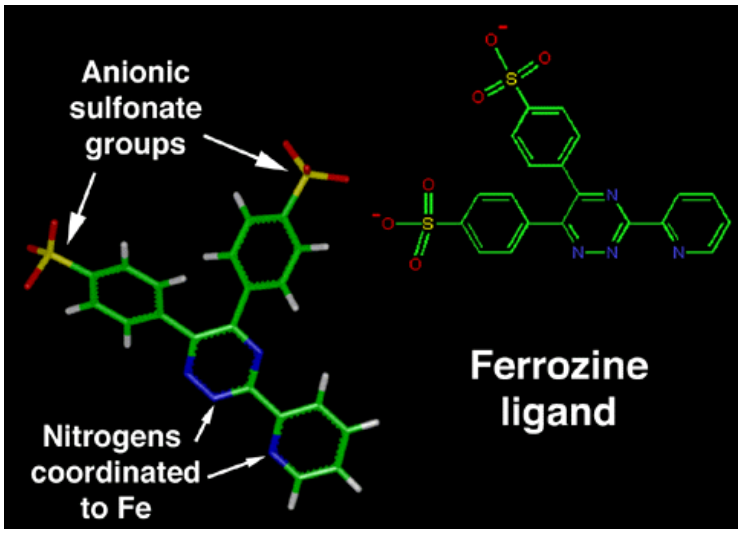


Figure 10.3: Chemical structure of the indicator ligand ferrozine. The left figure is a stick representation and the right figure is a ChemDraw® representation of ferrozine. The carbon atoms are green, the hydrogens are white, the nitrogen atoms are blue, the oxygen atoms are red, and the sulfur atoms are yellow. (http://www.chemistry.wustl.edu/~edudev/LabTutorials/Ferritin/ferrozine.html)

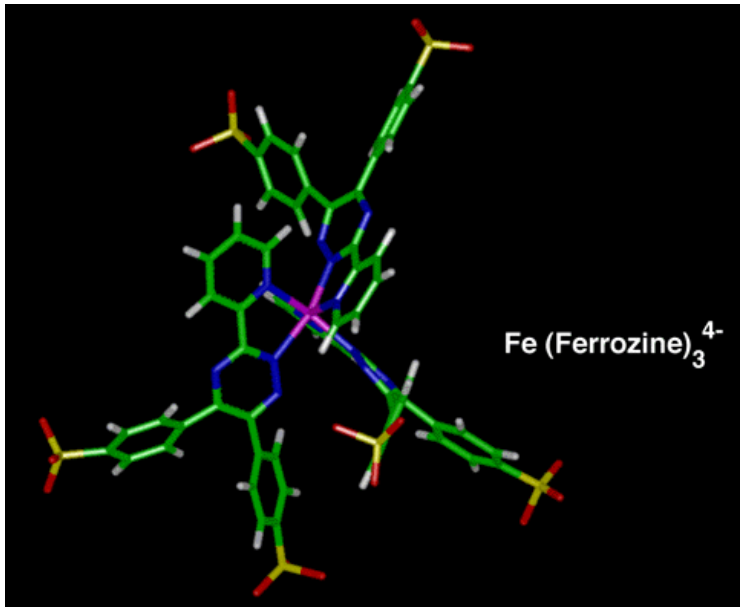


Figure 10.4: The Fe(II)-ferrozine complex where Fe^{2+} is complexed with three ferrozine ligands. The carbon atoms are green, the hydrogens are white, the nitrogen atoms are blue, the oxygen atoms are red, the sulfur atoms are yellow and the Fe^{2+} is purple. (http://www.chemistry.wustl.edu/~edudev/LabTutorials/Ferritin/feferr.html)

In the presence of compounds that also have the ability to chelate Fe^{2+}, the formation of the Fe(II)-ferrozine complex is inhibited leading to a decrease in color formation and a lower absorbance at 562 nm. The greater the Fe^{2+} chelating activity of a compound, the greater the inhibition of Fe(II)-ferrozine complex formation. Therefore, measuring the inhibition of Fe(II)-ferrozine complex formation may help to elucidate whether DA or 6-OHDA is able to bind Fe^{2+} and which ligand has the strongest affinity for Fe^{2+}.

10.2.2 MATERIALS AND METHODS

10.2.2.1 Chemicals and reagents

DA hydrochloride, 6-OHDA hydrobromide, ferrozine and horse spleen ferritin were purchased from Sigma Chemical Corporation, St Louis, MO, U.S.A. EDTA and ferrous sulfate were purchased from Merck, Darmstadt, Germany.

10.2.2.2 Chelating activity of DA and 6-OHDA

The Fe^{2+} chelating activity of DA and 6-OHDA was assessed by measuring the percentage (%) inhibition of the Fe(II)-ferrozine complex using the method of Decker and Welch (1990). Reaction mixture (final volume 5 ml) containing increasing concentrations (0.025, 0.05, 0.1, 0.2, 0.4 and 1 mM) of the test compound (DA or 6-OHDA), ferrous sulphate (0.04 mM) and ferrozine (0.2 mM) was incubated at room temperature for 20 minutes. The absorbance of the reaction mixture was then measured at 562 nm using a GBC 916 UV/VIS spectrophotometer. Lower absorbance of the reaction mixture indicated a higher chelating activity of the test compound being studied. The percentage inhibition of the Fe(II)-ferrozine complex formation was calculated using the following formula: % inhibition of the Fe(II)-ferrozine complex = $[(A_0 – A_1) / A_0] \times 100$, where A_0 is the absorbance of the control (reaction mixture without the test compound)

and A_1 is the absorbance in the presence of different concentrations of the test compound. EDTA, at the same concentrations as the test compounds was used as the positive control.

10.2.2.3 Iron release from ferritin assay

Iron reduction and release from ferritin was determined by the spectrophotometric measurement of the Fe(II)-ferrozine complexes (Babincová and Babinec, 2005). Reaction mixture containing ferritin (200 µg/ml), ferrozine (0.2 mM) and 6-OHDA (0.025, 0.05, 0.1, 0.2 and 0.4 mM) or DA (0.025, 0.05, 0.1, 0.2 and 0.4 mM) was incubated at 37 ºC in phosphate buffered saline (pH 7.4) for 20 minutes. The absorbance of the reaction mixture was then measured at 562 nm using a GBC 916 UV/VIS spectrophotometer. Higher absorbance of the reaction mixture at 562 nm indicated an increased release of iron, as Fe^{2+} from ferritin. Control incubations contained the same reaction mixture except that 6-OHDA (or DA) was not added to these incubations. The amount of iron released from ferritin by 6-OHDA and DA is expressed as a percentage of the control values.

10.2.3 RESULTS

Figure 10.5 shows that both DA and 6-OHDA are not capable of chelating Fe^{2+}. Even at a high concentration of 1 mM these drugs have no effect on the formation of the Fe(II)-ferrozine complex. EDTA was used as the positive control since it has a high affinity for iron (both ferrous and ferric iron) (Wong and Kitts, 2001). The high affinity of EDTA for Fe^{2+} makes it very difficult for any other chelator, including ferrozine to compete for iron binding. As a result of this EDTA is 100 % effective at inhibiting the formation of the purple Fe(II)-ferrozine complex at concentrations greater than 0.1 mM. Figure 10.6 shows that both DA and 6-OHDA lead to a concentration dependent release of iron, as Fe^{2+} from horse spleen ferritin. The 0.4 mM concentration of DA results in a 30 % increase in iron release from ferritin compared to the control values. The addition of

6-OHDA (0.4 mM) to control incubations increased the release of iron from ferritin by 183 %.

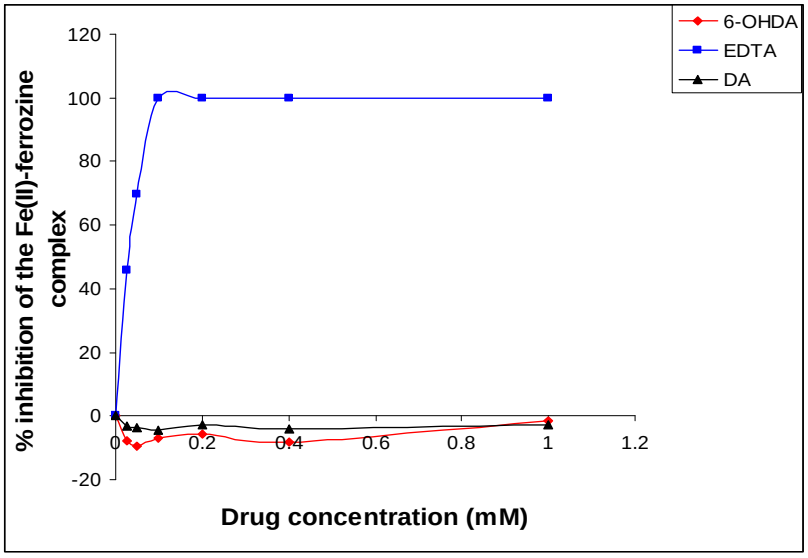


Figure 10.5: Percentage inhibition of the Fe(II)-ferrozine complex by DA, 6-OHDA and EDTA. Each point represents the mean ± SD (n = 5).

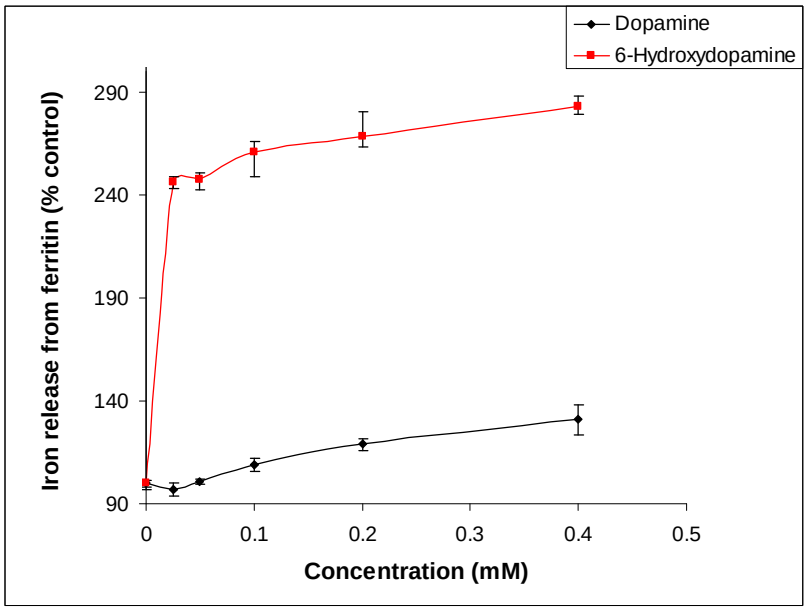


Figure 10.6: Release of iron from ferritin by DA and 6-OHDA expressed as a percentage of the control values. Each point represents the mean ± SD (n=5).

10.2.4 DISCUSSION

As brain iron is primarily bound to ferritin in the ferric form, its involvement in the Fenton reaction requires that it be reduced to its ferrous form on release from its binding site (Double *et al*., 1998). The results of the present study and in previous studies (Montinero and Winterbourne, 1989; Jellinger *et al*., 1995) show that both DA and 6-OHDA, or a species formed during the oxidation of these molecules is capable of releasing iron, as Fe^{2+} from horse spleen ferritin. These agents can therefore provide sufficient Fe^{2+} for Fenton chemistry to make •OH from H_2O_2 in the presence of ferritin. Hydroxyl radicals, in turn, can damage proteins, nucleic acids and membrane phospholipids, eventually leading to cellular degeneration. The mobilization of iron from ferritin has also been linked to changes in mitochondrial calcium homeostasis and subsequent GSH depletion and oxidative stress (Youdim and Riederer, 1993).

6-OHDA was found to be more effective at releasing Fe^{2+} from ferritin than was DA. This is probably related to the redox potential (the ability to donate and receive electrons) of these compounds. 6-OHDA is more readily oxidized than DA and is therefore more likely to donate an electron to Fe^{3+} to form Fe^{2+}. The manner in which these compounds interact with the Fe^{3+} in the protein shell of ferritin may also be an important factor with regard to the efficiency with which these agents release iron from ferritin. Linert *et al*., (1996) reported that 6-OHDA reacts with Fe^{3+} almost exclusively via an outer-sphere mechanism at physiological pH, i.e. electron exchange takes place without the prior formation of a metal-ligand complex. This is in contrast to DA which forms relatively stable bis- and tris- complexes with Fe^{3+} at physiological pH (Jameson *et al*., 2004). However, the findings of Linert *et al*. (1996) are in contrast to that of another study by Double *et al*. (1998), which shows that the ortho-dihydroxyphenyl group (catechol group) of 6-OHDA is necessary for iron release from ferritin because the first stage of the release process involves forming a Fe(III)-catechol complex. A following experiment will use adsorptive stripping voltammetry and cyclic voltammetry in order to investigate whether or not 6-OHDA forms a metal-ligand complex with Fe^{3+}. The following experiment will also determine the oxidation potentials of DA and 6-OHDA under the

same pH conditions (pH 7.4) in order to investigate the correlation between the redox potential of the compound and its ability to reduce ferritin iron.

The release of iron from ferritin by DA and 6-OHDA may be due to the reduction of Fe^{3+} to Fe^{2+} by the parent compound (Equation 10.1) or by $O_2^{\bullet-}$ (Equation 10.2) formed during the auto-oxidation of these compounds (Yoshida *et al*., 1995; Linert *et al*., 1996; Babincová and Babinec, 2005). Lode *et al*. (1990) found that 6-OHDA-induced Fe^{2+} release from ferritin is enhanced under anaerobic conditions. This suggests that it is the reduced form of 6-OHDA, and not $O_2^{\bullet-}$, that is responsible for 6-OHDA-induced iron release. On the other hand DA-induced Fe^{2+} release from ferritin is increased under high oxygen conditions and is reduced under low oxygen conditions (Double *et al*., 1998). This therefore suggests that the release of iron from ferritin by DA may be due to $O_2^{\bullet-}$ rather than DA itself. Claims that the reduction of the iron within ferritin can be carried out by DA itself is suspect because, at physiological pHs, the bis and tris-complexes of DA and Fe^{3+} predominate, and are remarkably stable to internal electron transfer (Jameson *et al*., 2004). Another possible mechanism for the release of iron from ferritin is protein damage induced by •OH. In the Fe^{2+} state, iron breaks away from the lattice and is released from the ferritin protein as soluble Fe^{2+} via the channels in the protein shell.

$$\text{Catechol} + Fe^{3+} \rightarrow SQ\bullet + Fe^{2+} \qquad \text{(Equation 10.1)}$$

$$O_2^{\bullet-} + Fe^{3+} \rightarrow O_2 + Fe^{2+} \qquad \text{(Equation 10.2)}$$

This study also shows that DA and 6-OHDA, even when used in relatively high concentrations (1 mM) are unable to chelate Fe^{2+} and inhibit the formation of the purple Fe(II)-ferrozine complex. The Fe^{2+} released from ferritin by 6-OHDA and DA would therefore be free to elicit free radical formation. The inhibition of lipid peroxidation induced by Fe^{2+} by DA and 6-OHDA can therefore not be explained by the chelation of Fe^{2+} by these compounds.

A following experiment will investigate the ability of DA and 6-OHDA to bind to Fe^{3+} and prevent its recycling to Fe^{2+}, the oxidation state necessary for its participation in the Fenton reaction.

10.3 EFFECT OF SELEGILINE ON THE FORMATION OF A FERROZINE-IRON (II) COMPLEX

10.3.1 INTRODUCTION

Although the etiology of PD is not yet well established, accumulating evidences have shown that iron-dependent oxidative stress, increased levels of iron and MAO-B activity and the depletion of antioxidants in the brain may be major pathogenic factors in PD (Zheng *et al*., 2005). A number of iron chelators (e.g. desferrioxamine) (Ben-Shachar *et al*., 1991), antioxidants (e.g. vitamin E) (Cadet *et al*., 1989) and MAO-B inhibitors (e.g. SEL) (Ebadi *et al*., 1996, Knoll, 1995) have been shown to possess neuroprotective activity and protect against the dopaminergic neurodegeneration induced by 6-OHDA (Zheng *et al*, 2005).

A single neuroprotective effect (iron chelation, free radical scavenging or MAO-B inhibition) may not be powerful enough to prevent neuronal death in PD. Neuroprotection in PD may therefore require a drug combining iron chelating with antioxidant and MAO-B inhibitory properties. SEL was the first highly selective inhibitor of MAO-B (Knoll and Magyar, 1972), and is the only selective MAO-B inhibitor in world wide clinical use (Knoll, 1995). Chapter 5 and 6 have shown that SEL also possesses antioxidant properties, both *in vitro* and *in vivo* by reducing the formation of •OH and $O_2^{\bullet-}$. The main aim of this study is to investigate whether SEL possesses any iron chelating properties.

10.3.2 MATERIALS AND METHODS

10.3.2.1 Chemicals and reagents

SEL hydrochloride was purchased from Sigma Chemical Corporation, St Louis, MO, U.S.A. All other reagents were purchased as described in section 10.2.2.1.

10.3.2.2 Chelating activity of SEL

The Fe^{2+} chelating activity of SEL was assessed by using the method of Decker and Welch (1990) described in section 10.2.2.2. Reaction mixture (final volume 5 ml) containing increasing concentrations (0.025, 0.05, 0.1, 0.2, 0.4 and 1 mM) of SEL, ferrous sulphate (0.04 mM) and ferrozine (0.2 mM) was treated as described in section 10.2.2.2.

10.3.3 RESULTS

Figure 10.7 shows that SEL results in a 20 % inhibition of the Fe(II)-ferrozine complex. However, the inhibition of Fe(II)-ferrozine complex formation by SEL does not appear to be concentration dependent. SEL is also less potent at chelating Fe^{2+} than is EDTA. At concentrations between 0.1 and 1 mM, EDTA results in complete inhibition of Fe(II)-ferrozine formation while SEL still only causes approximately 20 % inhibition between these concentrations.

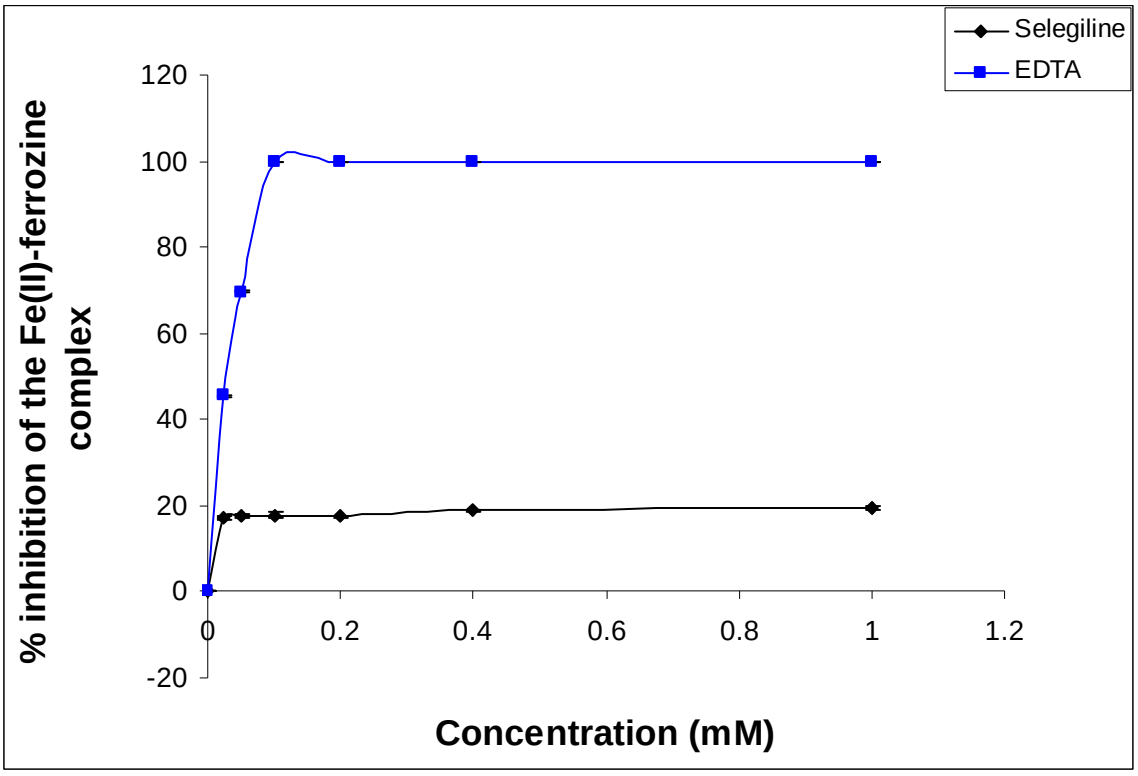


Figure 10.7: Percentage inhibition of the Fe(II)-ferrozine complex by SEL and EDTA. Each point represents the mean ± SD (n = 5).

10.3.4 DISCUSSION

Chelating agents are usually flexible molecules with two or more electronegative functional groups (e.g. –OH, -SH and –NH) that form a covalent bond with cationic metal atoms. The structure of SEL (figure 10.8) does not therefore fit the mold of a typical iron chelator since it only has one electronegative atom (nitrogen) in its structure.

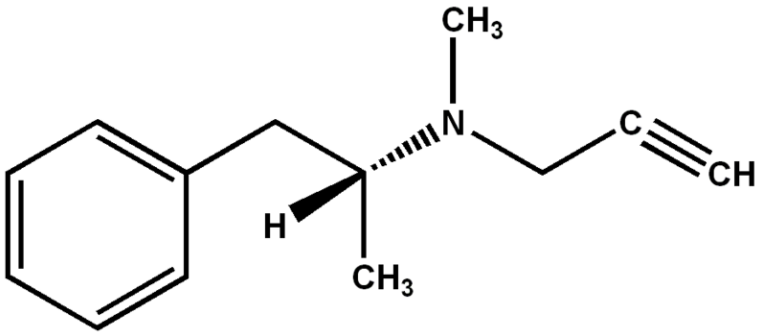


Figure 10.8: Structure of SEL.
(http://commons.wikimedia.org/wiki/Image:Selegiline-structure.png)

The results of this study suggest that SEL has the capacity to bind Fe^{2+} ions. The results also demonstrate that SEL is a relatively weak iron binder compared to EDTA, which was used as the positive control. The acetylenic group (alkyne group) of the propargyl moiety of SEL is the functional group most likely to be involved in iron binding. Alkynes are electropositive and tend to bind transition metals more strongly than alkenes. Alkynes have two sets of mutually orthogonal pi bonds and can bind to transition metals in a sigma-type fashion and in a pi-type fashion (Garnovskii *et al*., 2003).

The propargyl moiety is believed to be responsible for the potent MAO-B inhibitory activity of SEL (Zheng *et al*., 2005). N-propargyl-containing compounds such as rasagiline, pargyline and clorgyline display high MAO inhibitory activity. However, removal of the propargyl moiety abolishes the inhibitory activity of these compounds (Youdim, 1978; Youdim and Weinstock, 2002). The binding of iron by the acetylenic group of SEL may therefore affect the MAO-B inhibitory activity of this compound. The

effect of iron binding on the MAO-B inhibitory activity of SEL therefore requires further investigation.

10.4 INTERACTION BETWEEN DOPAMINE, 6-HYDROXYDOPAMINE AND FERRIC IRON: AN ELECTROCHEMICAL STUDY

10.4.1 INTRODUCTION

A chemical reaction in which electrons are transferred from one species to another (redox reactions) is the basis for electrochemical analysis (Pecsok *et al*., 1968). Most electrochemical cells used for electroanalytical measurements utilize three electrodes, namely, a working, a reference and an auxiliary (or counter) electrode (Bard and Faulkner, 1980), as depicted in figure 10.9.

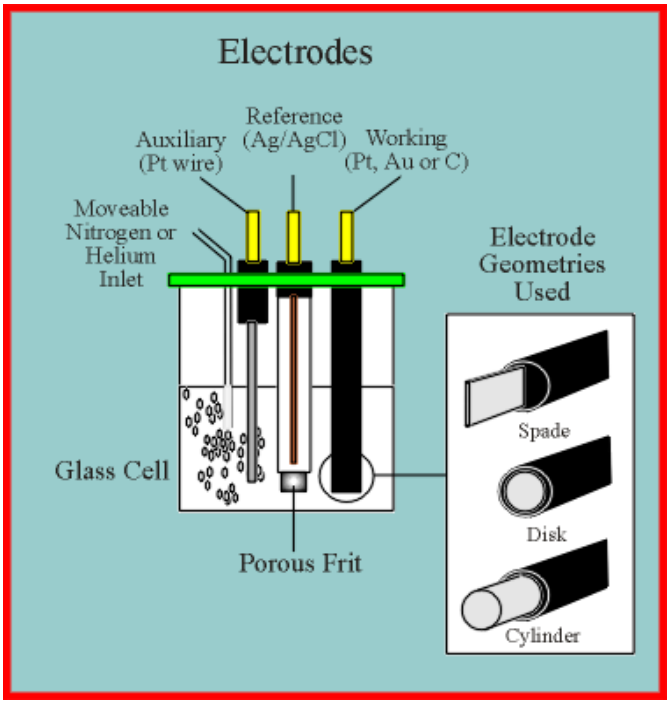


Figure 10.9: Most electrochemical cells utilize a three electrode system as shown here. (http://www-biol.paisley.ac.uk/marco/Enzyme_Electrode/Chapter1/Ferrocene_animated_CV1.htm)

The working electrode is the electrode at which the analyte is oxidized or reduced i.e. electron transfer reactions occur at the surface of this electrode. The reference electrode maintains a constant potential and serves as a reference standard against which the potential of the working electrode can be measured. The auxiliary electrode serves as a source or sink of electrons so that current can be passed from the external circuit through the cell (Bard and Faulkner, 1980).

In the following experiments two electroanalytical techniques, adsorptive stripping voltammetry (AdSV) and cyclic voltammetry (CV) were employed to study the ability of DA and 6-OHDA to bind Fe^{3+} under de-aerated conditions. The study was conducted in aqueous media and under biological pH conditions.

10.4.1.1 Adsorptive stripping voltammetry

Adsorptive stripping voltammetry (AdSV) exploits the natural tendency of analytes to pre-concentrate at an electrode and is a useful electrochemical technique for studying metal-ligand interactions (Limson, 1998).

The first step in AdSV is the reaction of the metal ion to be studied with a suitable ligand leading to the formation of a metal-ligand complex (equation 10.3) (Limson *et al*., 1998). This is followed by the controlled interfacial accumulation (adsorption) of the metal-ligand complex onto the electrode at a fixed deposition potential (equation 10.4) (Limson *et al*., 1998). The third step is the electrochemically active stripping step (Limson *et al*., 1998). It involves the reduction of the adsorbed metal-ligand complex by application of a potential in the negative direction, releasing the metal and ligand back into solution (equation 10.5) (Limson *et al*., 1998). The flow of electrons during the last step produces a cathodic current which peaks at the reduction potential of the complex. The current produced is a direct measure of the rate at which reduction occurs (Limson *et al*., 1998). The resulting plot of current versus potential is called a voltammogram.

$$M^{n+} + x(Ligand)_{aq} \rightarrow M(Ligand)_{x,aq} + xnH^{+} \quad \text{(Equation 10.3)}$$

$$M(Ligand)_{x,aq} \rightarrow M(Ligand)_{x,ads} \quad \text{(Equation 10.4)}$$

$$M(Ligand)_{x,ads} + xnH^{+} + ne^{-} \rightarrow M^{n+} + x(Ligand)_{aq} \quad \text{(Equation 10.5)}$$

A metal species at an electrode will produce a characteristic voltammogram at a specific potential, with current being proportional to the amount of analyte at the electrode (Limson, 1998). When a suitable ligand is added to a metal solution, the ligand facilitates movement of the metal to the electrode (Limson, 1998). Theoretically then, the binding of a metal to a ligand should bring about an increase in the current response observed. The extent of the increase in current response of the metal on addition of the ligand is an indication of the affinity of the ligand for the metal (Limson *et al*, 1998). A lowering in the current response at relatively high analyte concentrations indicates possible competition between the free ligand and the metal-ligand complex for binding sites at the surface of the electrode (Limson, 1998). A decrease in current response at low ligand concentrations is more likely due to the formation of a strong metal-ligand complex that is not easily reduced to produce a cathodic current (Limson, 1998).

The formation of a metal-ligand complex may also cause a shift in potential resulting from the reduction of a new species at the electrode. The extent of the shift in the reduction potential of the metal after the formation of a metal-ligand complex is an indication of the stability of the metal-ligand complex (Limson *et al*., 1998). A negative potential shift indicates the formation of strong metal-ligand complex (which has a lower tendency to become reduced) while a large positive shift is associated with the formation of a weaker metal-ligand complex (Limson, 1998).

10.4.1.2 Cyclic voltammetry

Cyclic voltammetry (CV) measurements have been used for many years to evaluate electron transfer reactions between molecules or species in solution (Kohen *et al*., 2000). In these techniques, an electrical potential gradient is applied (relative to the reference electrode) across the electrode-solution interface (working electrode) to oxidize or reduce species present in solution (a linear potential gradient is applied in the case of CV) (Kohen *et al*., 2000; Ligumsky *et al*., 2005). The operation of the voltammeter results in a recording of the potential versus current curve, cyclic voltammogram (figure 10.10). The voltammogram provides information concerning the thermodynamics, kinetics, and analytical features of the electrochemical species under investigation (Kohen *et al*., 2000; Ligumsky *et al*., 2005). Although many factors determine the shape and value of the current wave (form of applied potential, electrode size and geometry, size of the energy barrier for electron transfer, interaction between the electrode surface, and the electroactive molecules), voltammetric waves are usually obtained in a peak shape or sigmoidal mode (Kohen *et al*., 2000). The position of the current wave on the voltage axis (the x-axis of the voltammogram) can be determined and is referred to as the potential where the peak current (peak potential – Ep(a)) or inflection point (half-wave potential, $E_{1/2}$) occurs ((Kohen *et al*., 2000; Ligumsky *et al*., 2005). The oxidation potential of a compound is defined as the potential where Ep(a) is observed for a given set of conditions (Kohen *et al*., 2000). The peak potential location on the x-axis (specific ability of the compound to donate electrons) reflects the nature or type of the compounds present in the sample (Ligumsky *et al*., 2005). The size of the anodic current (AC, Ia), calculated from the y-axis of the voltamogram, is proportional to the concentration of the substrate in the bulk solution (Bard and Faulkner, 1980). These two parameters (peak potential and AC) are characterized as the reducing power parameter and can supply information regarding the type of reducing antioxidants (their ability to donate electrons) and their total concentration (Kohen *et al*., 2000).

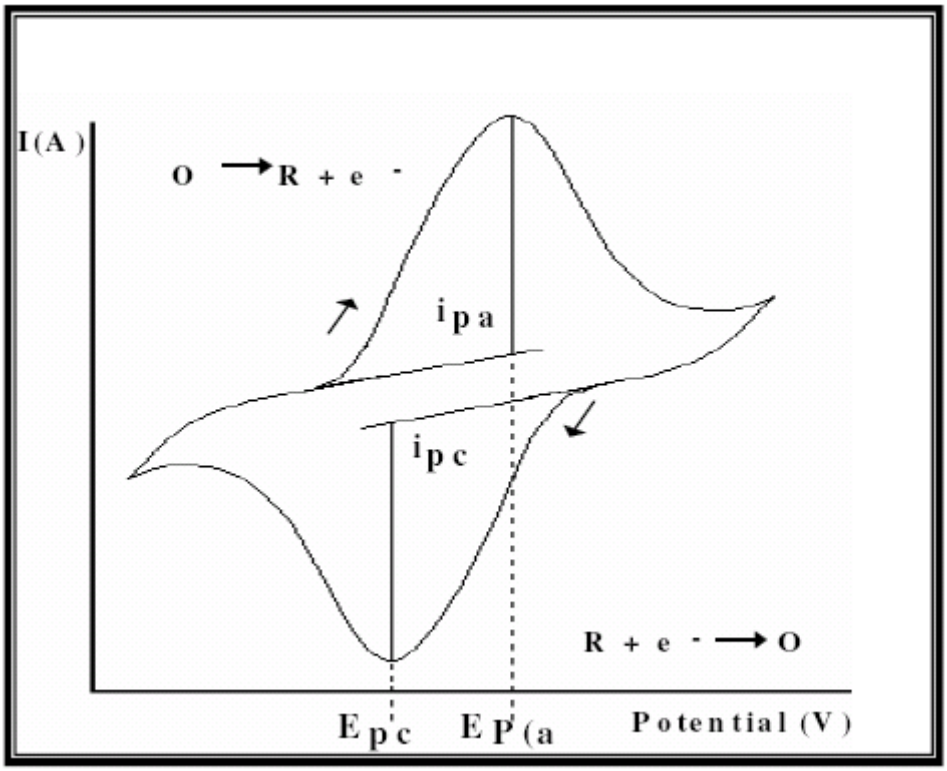


Figure 10.10: A typical cyclic voltammogram (Limson, 1998).

Electroactive species produce characteristic redox patterns in solution. Changes in the current intensity and the redox behaviour of the redox waves provide insight into the interaction of species in solution. Redox couples are typically located by potential scans encompassing the entire accessible window period of the electrode (Limson, 1998). The forward scan generates the oxidized species and the reverse scan the reduced species (Limson, 1998). Changes in the potential and current response are good indicators of alterations in the chemistry of the electroactive species in solution, such as that which occurs during an exchange of electrons in a metal-ligand bond.

10.4.2 MATERIALS AND METHODS

10.4.2.1 Chemicals and reagents

DA hydrochloride, 6-OHDA hydrobromide and Tris-HCl were purchased from Sigma Chemical Corporation, St Louis, MO, U.S.A. Anhydrous ferric chloride ($FeCl_3$) was purchased from Merck, Darmstadt, Germany.

10.4.2.2 Instrumentation

Adsorptive stripping voltammograms and cyclic voltammograms were recorded on an Autolab PGSTAT 30 voltammeter equipped with a Metrohm VGA cell stand. A 3 mm glassy carbon electrode (GCE) was employed as the working electrode for the voltammetric experiments. A silver/silver chloride (Ag/AgCl) [(KCl = 3 M)] and a platinum wire were used as the reference and auxiliary electrodes, respectively, in all the voltammetric experiments. Prior to use and between scans, the GCE was cleaned and polished with alumina on a Buehler pad, followed by immersion in dilute nitric acid solution and rinsing in Milli-Q water.

10.4.2.3 Adsorptive stripping voltammetry

An appropriate concentration of $FeCl_3$ was introduced into the electrochemical cell containing the electrolyte, 0.2 M Tris-HCl, pH 7.4. The electrolyte was then deaerated with nitrogen for 5 minutes with simultaneous solution stirring. The reason for deaerating the solution is to minimize the interference of oxygen with the electrochemical measurements. Thereafter, an optimum deposition potential for the Fe^{3+} was identified and applied for 60 seconds to affect the adsorption of the metal ion onto the surface of the GCE. A potential scan in the negative direction from the deposition potential to at least 0.5 V beyond the reduction potential of the metal was applied, at a scan rate of 0.1 $V.s^{-1}$,

to strip the adsorbed species from the GCE. During the stripping step, current responses due to the reduction of the metal species were measured as a function of potential. The GCE was then cleaned and polished as described in section 10.4.2.2. The procedure was repeated between successive additions of appropriate concentrations of either DA or 6-OHDA (0 – 0.06 mM) to the electrolyte containing the Fe^{3+} in the electrochemical cell.

Current versus concentration and potential versus concentration plots were constructed to measure the extent of shifts in current response and reduction potential of metal with increasing concentrations of the ligand (either DA or 6-OHDA).

10.4.2.4 Cyclic voltammetry

For the cyclic voltammetric experiments, appropriate concentrations of 6-OHDA or DA were added to the electrochemical cell containing 0.2 M Tris-HCl, pH 7.4 as the electrolyte. The electrolyte was then degassed for 5 minutes with nitrogen to prevent interference by any oxygen effects. A potential window was then scanned to characterize and provide a fingerprint of the species in solution. The GCE was then cleaned and polished as described in section 10.4.2.2. The procedure was repeated between successive additions of appropriate concentrations of Fe^{3+} to the electrolyte containing either DA or 6-OHDA in the electrochemical cell.

Changes in current response and potential were observed in order to investigate whether 6-OHDA and DA were binding the Fe^{3+} ions to form a new species (metal-ligand complex) in the solution.

10.4.3 RESULTS

10.4.3.1 Adsorptive stripping voltammetry

Figure 10.11 shows the adsorptive stripping voltammogram for Fe^{3+} in solution, with a peak current response of 2.10 x 10^{-6} A and a reduction potential of -0.39 V. The addition of increasing concentrations of DA (0.01 – 0.06 mM) to the electrochemical cell causes a concentration dependent-shift in the reduction potential of Fe^{3+} towards more negative potentials (figure 10.12) and a decrease in the current response (figure 10.13).

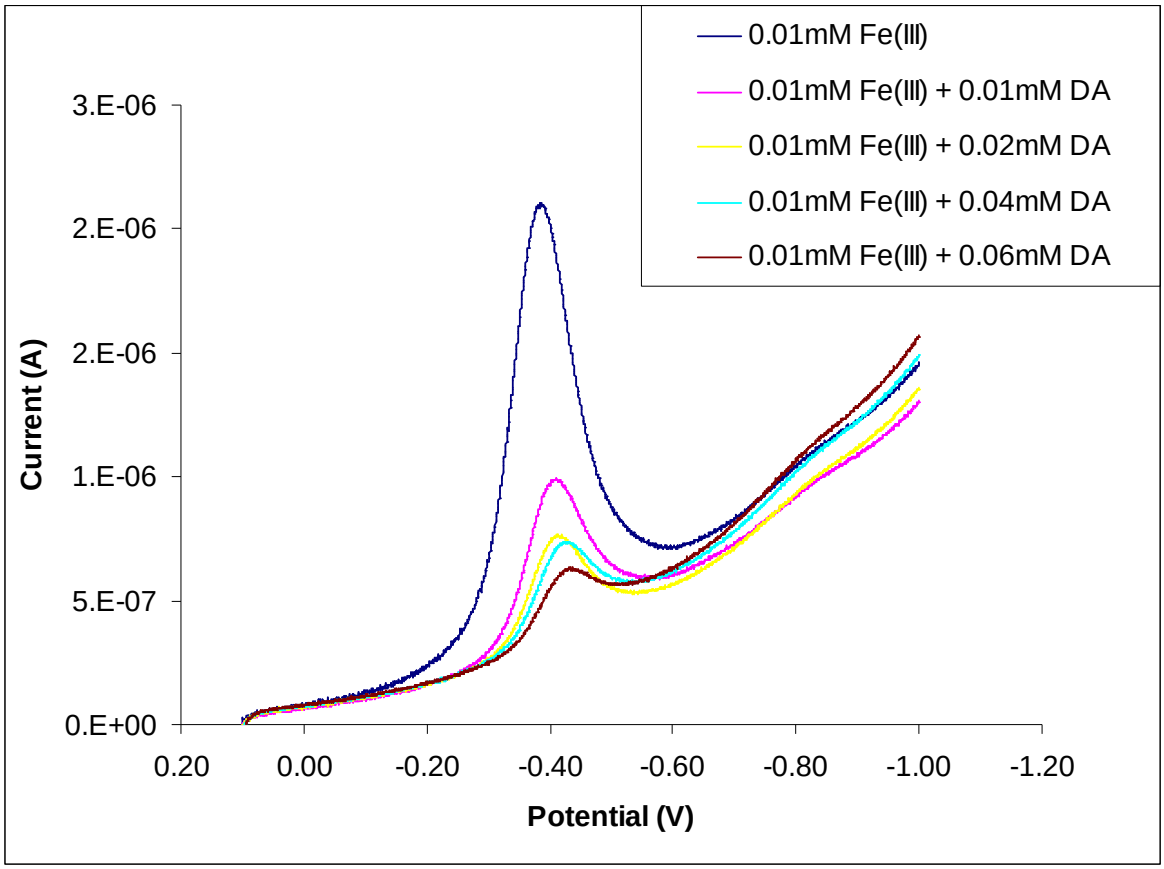


Figure 10.11: Adsorptive stripping voltammogram for Fe^{3+} (0.01 mM) alone and in the presence of increasing concentrations of DA (0.01 – 0.06 mM).

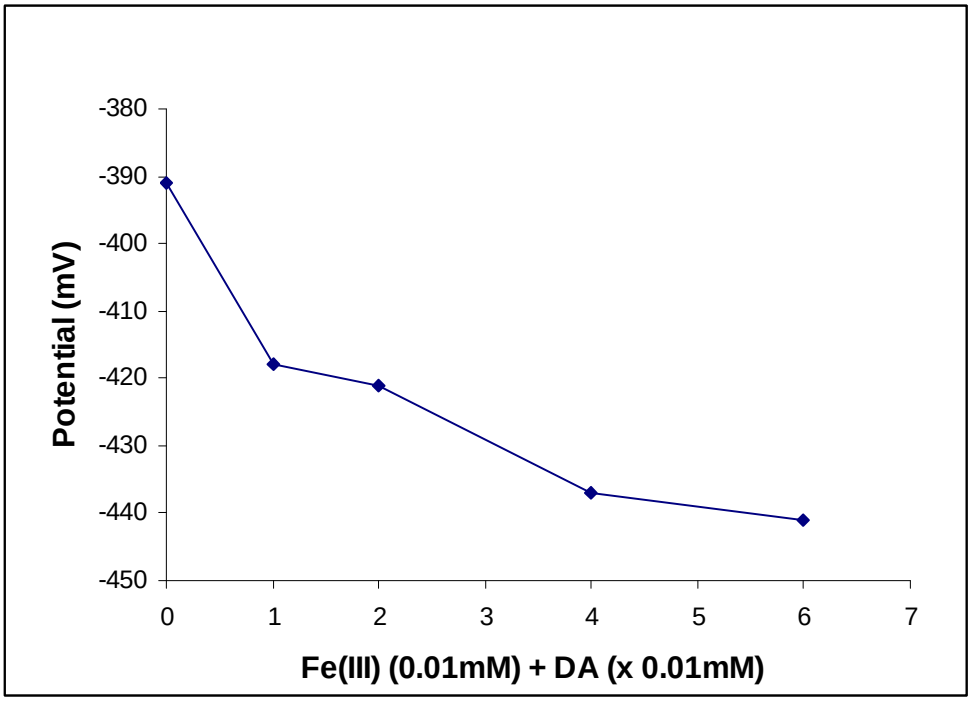


Figure 10.12: Effect of increasing concentrations of DA on the reduction potential of 0.01 mM Fe^{3+}.

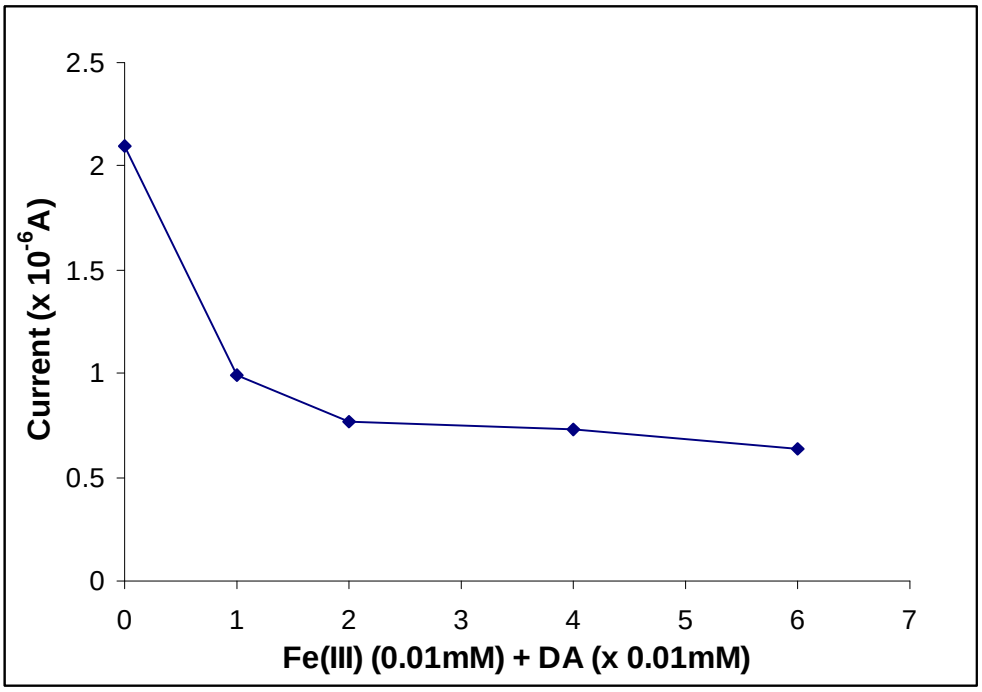


Figure 10.13: Effect of increasing concentrations of DA on the peak current response of 0.01 mM Fe^{3+}.

The adsorptive stripping voltammogram of Fe^{3+} in the presence of increasing concentrations of 6-OHDA is depicted in figure 10.14. The peak formed at -0.29 V is attributed to 6-OHDA. A scan of 6-OHDA alone shows its reduction potential in the same region. Initially there is a decrease in current response and a small shift to more negative potentials at a metal to ligand ratio of 1:1 and 1:2. However, at higher concentrations of 6-OHDA the peak attributed to 6-OHDA interferes strongly with the Fe^{3+} peak. The results of this voltammetric experiment are therefore not conclusive (due to the interference of the 6-OHDA peak). For this reason cyclic voltammetry was used to confirm whether or not 6-OHDA has the ability to bind Fe^{3+}.

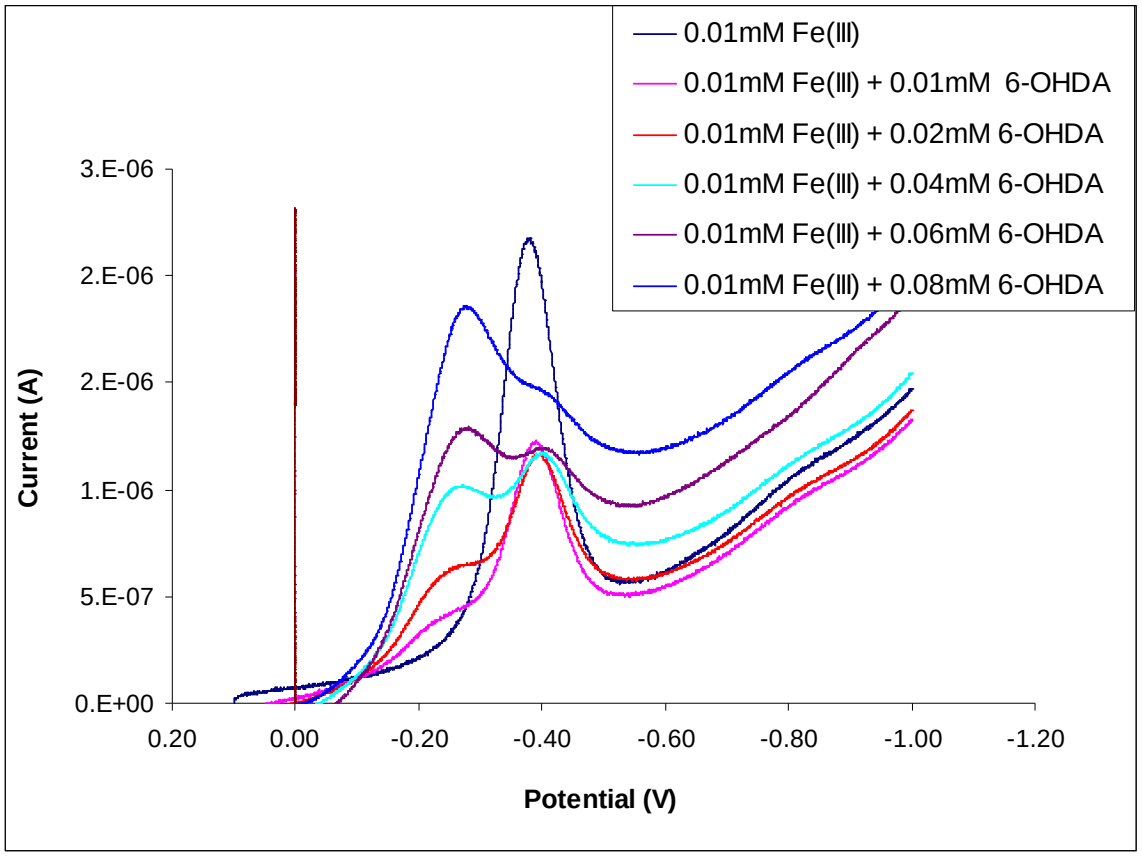


Figure 10.14: Adsorptive stripping voltammogram for Fe^{3+} (0.01 mM) alone and in the presence of increasing concentrations of 6-OHDA (0.01 – 0.08 mM).

10.4.3.2 Cyclic voltammetry

Figure 10.15 presents a series of cyclic voltammograms of dopamine in the presence of increasing concentrations of Fe^{3+}. In figure 10.15, peak 1 (0.17 V) and 2 (0.11 V) are assigned to the DA redox peaks. Peak 3 is most likely the redox peak of one of the oxidation products of DA. Peak 3 has a reduction potential of -0.28 V, similar to that observed for dopaminochrome (Wang *et al.*, 2006). Compared to the cyclic voltammogram obtained with DA alone, an increase in the concentration of Fe^{3+} caused a rapid and concentration-dependent decrease in peak 1 on the forward scan and a rapid decrease in peaks 3 and 4 on the reverse scan. The DA peak (peak 1) also shifts to a more positive potential with increasing Fe^{3+} concentration. The cyclic voltammogram of DA strongly suggests that DA has the ability to bind Fe^{3+} to form a metal-ligand complex and confirms the results of the AdSV study.

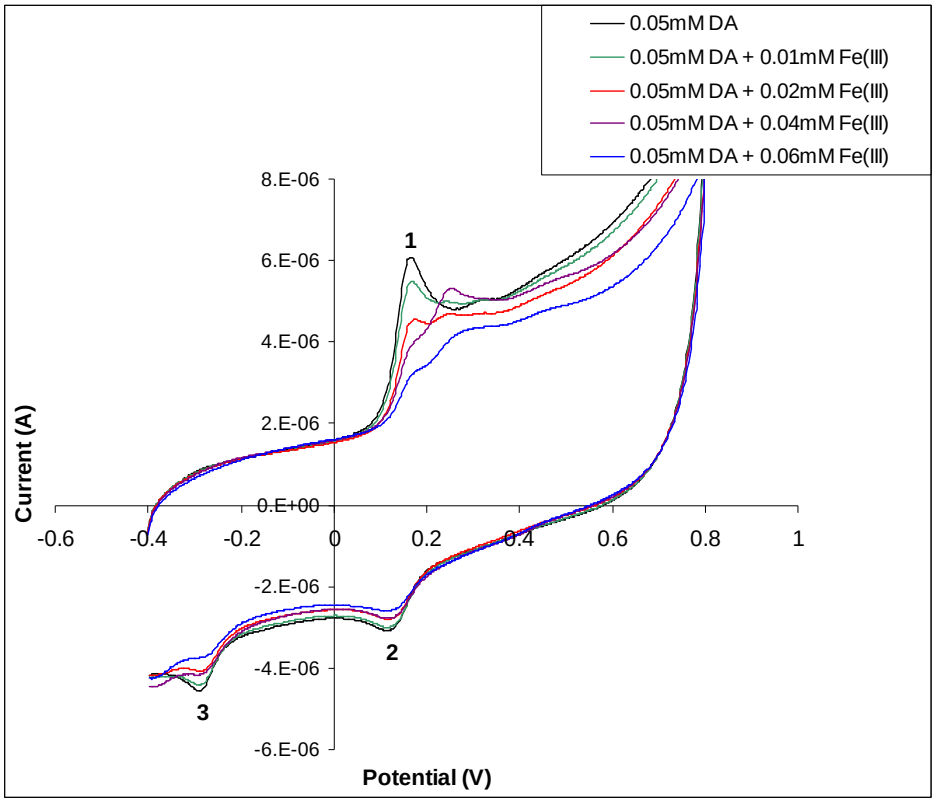


Figure 10.15: Cyclic voltammogram of DA alone and in the presence of increasing concentrations of Fe^{3+}.

Figure 10.16 presents a series of cyclic voltammograms of 6-OHDA alone and in the presence of increasing concentrations of Fe^{3+} (0 - 0.06 mM). 6-OHDA is electrochemically active at the GCE, meaning that redox waves are observed for this compound. In figure 10.16, peak 1 (-0.15 V), the anodic wave and peak 2 (-0.20 V), the cathodic wave are assigned to the 6-OHDA redox peaks. An increase in the concentration of Fe^{3+} had no effect on peak 1 during the forward scan (no significant change in current response or potential) and caused a small increase in the current response of peak 2 during the reverse scan without any observable potential shift. The peak formed at -0.40 V (peak 3) is attributed to Fe^{3+}. A scan of Fe^{3+} alone shows its reduction potential in the same region. The cyclic voltammogram in figure 10.16 therefore provides no evidence for the formation of a metal-ligand complex between 6-OHDA and Fe^{3+} in solution.

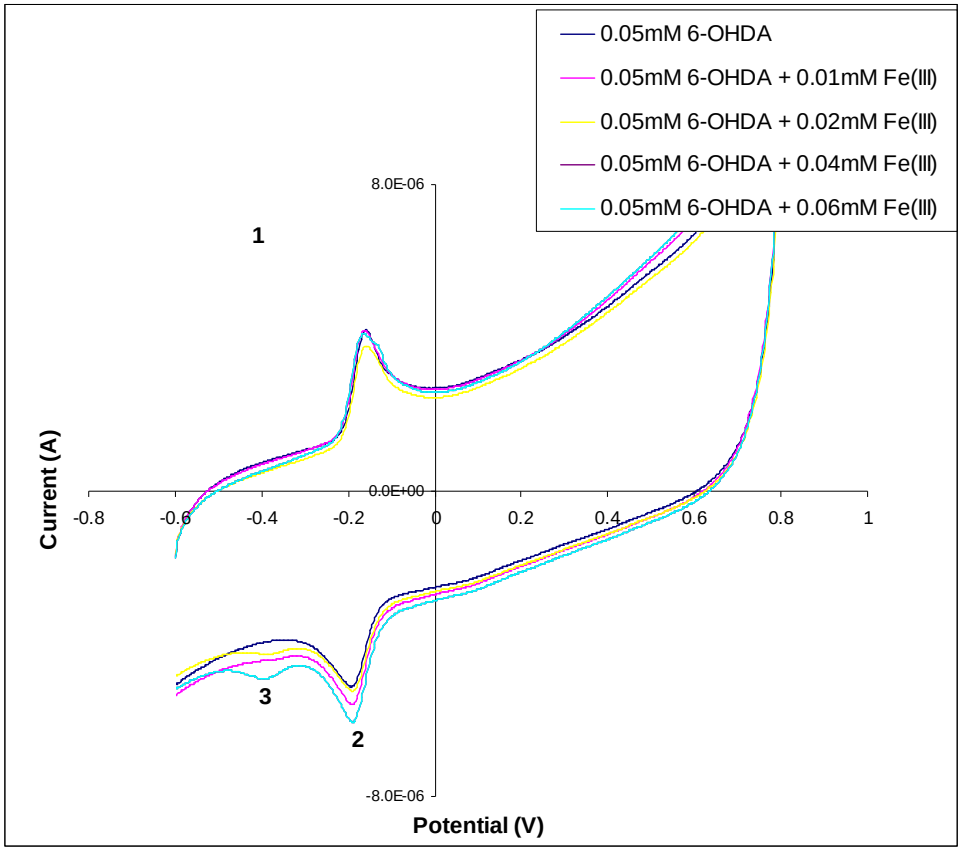


Figure 10.16: Cyclic voltammogram of 6-OHDA alone and in the presence of increasing concentrations of Fe^{3+}.

10.4.4 DISCUSSION

The reactivity of iron varies greatly depending on the environment that the chelator provides (Welch *et al*., 2002). In general, iron is liganded by electronegative atoms, particularly by oxygen, nitrogen and sulphur atoms. Chelators affect the reduction potential of iron, i.e. the ease with which iron is reduced. Oxygen ligands prefer Fe^{3+}, thus the reduction potential of the ligand is usually decreased (Miller *et al*., 1990). Conversely, nitrogen and sulphur ligands prefer Fe^{2+}, thus the reduction potential of the iron is usually increased (Miller *et al*., 1990).

Therefore, chelators with oxygen atoms, such as DA tend to inhibit the reduction of Fe^{3+} to Fe^{2+}. This is consistent with the results of the present electrochemical study. The AdSV of Fe^{3+} in the presence of increasing concentrations of DA (figure 10.11) shows that DA has the ability to form a complex with Fe^{3+} which has a more negative reduction potential than Fe^{3+} alone. Therefore, DA forms a complex with Fe^{3+} that is more difficult to reduce to Fe^{2+} than Fe^{3+} alone and is therefore likely to curtail the reduction of Fe^{3+} to Fe^{2+}, the form of iron required for the Fenton reaction. The shift in the reduction potential of the metal after the formation of a metal-ligand complex is an indication of the stability of the metal-ligand complex (Limson *et al*., 1998). Figure 10.12 shows that there is a relatively large shift in the reduction potential of Fe^{3+} as the concentration of DA increases, this indicates that a relatively stable complex has been formed. Since low ligand concentrations were used during the present study, the decrease in the current response of Fe^{3+} after the formation of a Fe(III)-DA complex (figures 10.11 and 10.13) indicates that a greater potential is required for the reduction of the complex, than that of the free metal species. This implies that the Fe(III)-DA complex is strong enough to inhibit the cathodic current flow. This is expected for the Fe(III)-DA complex since it has a more negative reduction potential than Fe^{3+} alone and is more difficult to reduce than the free metal ion.

Ferric-stabilizing chelators are protective if these prevent Fe^{3+} from being recycled to Fe^{2+}. The chelation of Fe^{3+} by dopamine suggests that the increase in •OH production on

addition of DA to the Fenton system (chapter 6) is probably not due to the involvement of Fe^{2+} in the production of •OH by the Fenton reaction. Thus, the observed effect is probably a consequence of the reported capacity of Fe^{3+} to catalyze the auto-oxidation of DA, leading to increased •OH production (Linert and Jameson, 2000). The ability of DA to bind Fe^{3+} does not therefore reduce •OH production by the Fenton system (chapter 6), however the binding of this metal ion may help to inhibit iron-induced lipid peroxidation via another mechanism. A possible mechanism in which iron could be involved in the intiation of lipid peroxidation involves the formation of a Fe(III)-Fe(II) complex. This mechanism has been proposed by many researchers (Bucher *et al*., 1983; Tang and Shen, 1997; Djuric *et al*., 2001). The inhibition of iron-induced lipid peroxidation by DA (chapter 7) may, at least in part, be due to the ability of DA to alter the ratio of Fe^{2+} to Fe^{3+} and not by scavenging or inhibiting •OH formation. Despite the inhibition of iron-induced lipid peroxidation, DA actually accelerates damage to proteins in the presence of iron. This finding only makes sense if the oxidation of these biomolecules occurs via different mechanisms (see chapter 8 for the possible mechanisms by which DA increases protein oxidation). If the oxidation of proteins and lipids occurs via the same mechanism, their oxidation would be inhibited or stimulated by the same chemicals (Welch *et al*., 2002).

The AdSV of Fe^{3+} in the presence of increasing concentrations of 6-OHDA did not give reliable information regarding whether or not 6-OHDA forms a metal-ligand complex with Fe^{3+} since the 6-OHDA peak interfered strongly with the Fe^{3+} peak. For this reason, cyclic voltammetry was used in order to investigate whether any binding took place between these two species in solution. Figure 10.16 shows that there was very little change in the cyclic voltammogram of 6-OHDA in the presence of increasing concentrations of Fe^{3+}. This is indicative of no complex formation between 6-OHDA and Fe^{3+}. This finding is therefore in agreement with the study of Linert *et al*. (1996) that proposed that 6-OHDA interacts with Fe^{3+} via an outer sphere electron transfer reaction without the prior formation of a 6-OHDA-Fe(III) complex. The inhibition of iron-induced lipid peroxidation by 6-OHDA can therefore not be explained by the chelation of Fe^{2+} or Fe^{3+}, or the scavenging of •OH and must occur via some other mechanism (chapter 7).

The cyclic voltammogram of DA showed significant shifts in both current response and potential for the DA redox peaks. This strongly implies complex formation between DA and Fe^{3+} and confirms the result of the AdSV experiment.

The cyclic voltammogram of 6-OHDA and DA show that the oxidation potential (Ep(a)) of 6-OHDA is around -160 mV and that of DA is approximately 176 mV at pH 7.4. The lower oxidation potential of 6-OHDA may account for its enhanced ability to release iron from ferritin when compared to DA.

10.5 INTERACTION BETWEEN SELEGILINE AND FERRIC IRON: AN ELECTROCHEMICAL STUDY

10.5.1 INTRODUCTION

The results of the ferrozine assay in section 10.3 demonstrate that SEL has weak Fe^{2+} chelating activity. SEL was able to inhibit Fe(II)-ferrozine complexation more effectively than DA or 6-OHDA, this finding is surprising considering that SEL has only one electronegative N atom in its structure. It was postulated that the alkyne group in the SEL molecule was responsible for the chelation of Fe^{2+}. In addition to MAO-B and antioxidant activity, SEL may possess some metal chelating activity. The present investigation was performed in order to investigate whether an interaction exists between SEL and Fe^{3+} in solution using adsorptive stripping voltammetry.

10.5.2 MATERIALS AND METHODS

10.5.2.1 Chemicals and reagents

SEL hydrochloride was purchased from Sigma Chemical Corporation, St Louis, MO, U.S.A. All other chemicals were purchased as described in section 10.4.2.1.

10.5.2.2 Instrumentation

As described in section 10.4.2.2.

10.5.2.3 Adsorptive stripping voltammetry

The procedure outlined in section 10.4.2.3 was followed, except that increasing concentrations of SEL, instead of DA or 6-OHDA, were added to the Fe^{3+} solution in the electrochemical cell between scans.

10.5.3 RESULTS

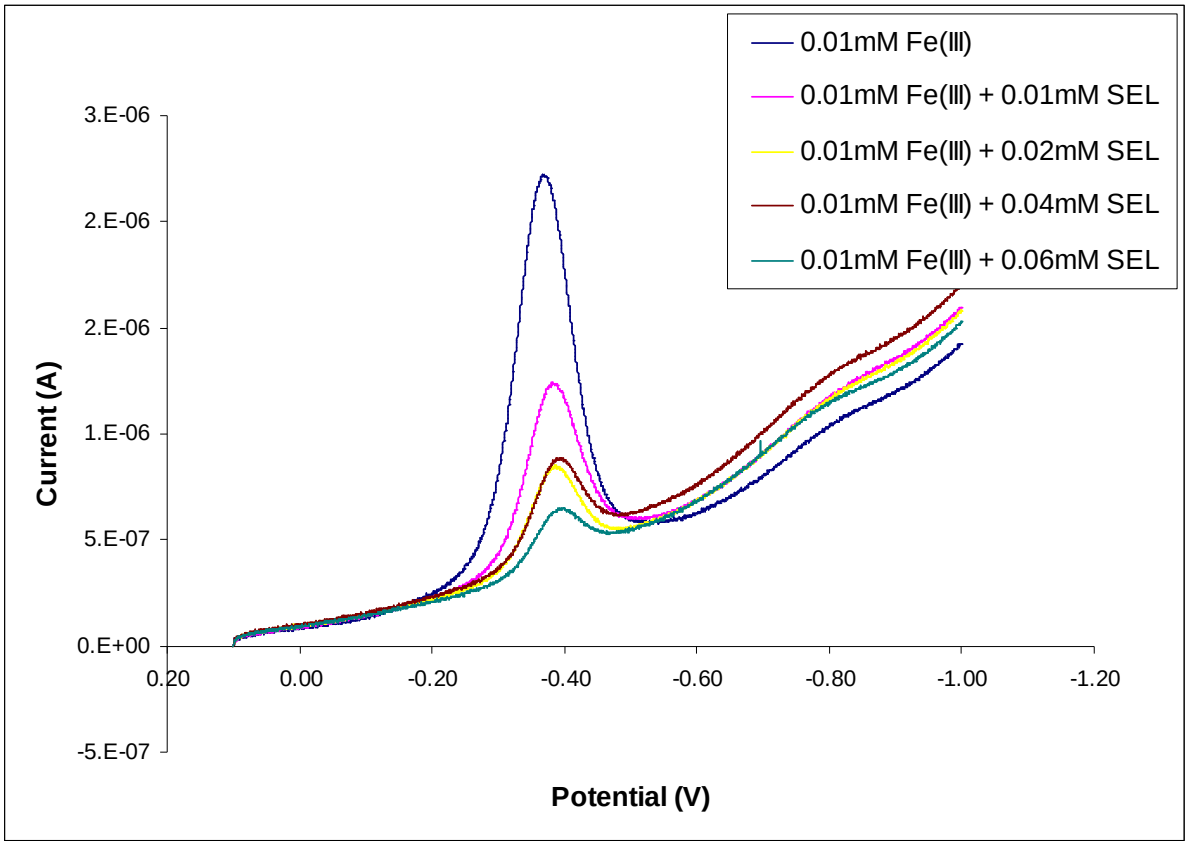


Figure 10.17: Adsorptive stripping voltammogram of Fe^{3+} alone and in the presence of increasing concentrations of SEL (0.01 – 0.06 mM).

In figure 10.17, the AdSV for 0.01 mM Fe^{3+} alone in 0.2 M Tris HCl, pH 7.4 buffer shows a peak current response of 2.20 x 10^{-6} A at a reduction potential of -0.38 V. The addition of increasing concentrations of SEL causes a significant and concentration-dependent shift in the reduction potential, to more negative potentials, and a decrease in current response. Figures 10.18 and 10.19 illustrate the concentration dependent decrease in the reduction potential and peak current response of 0.01 mM Fe^{3+} in the presence of increasing concentrations of SEL.

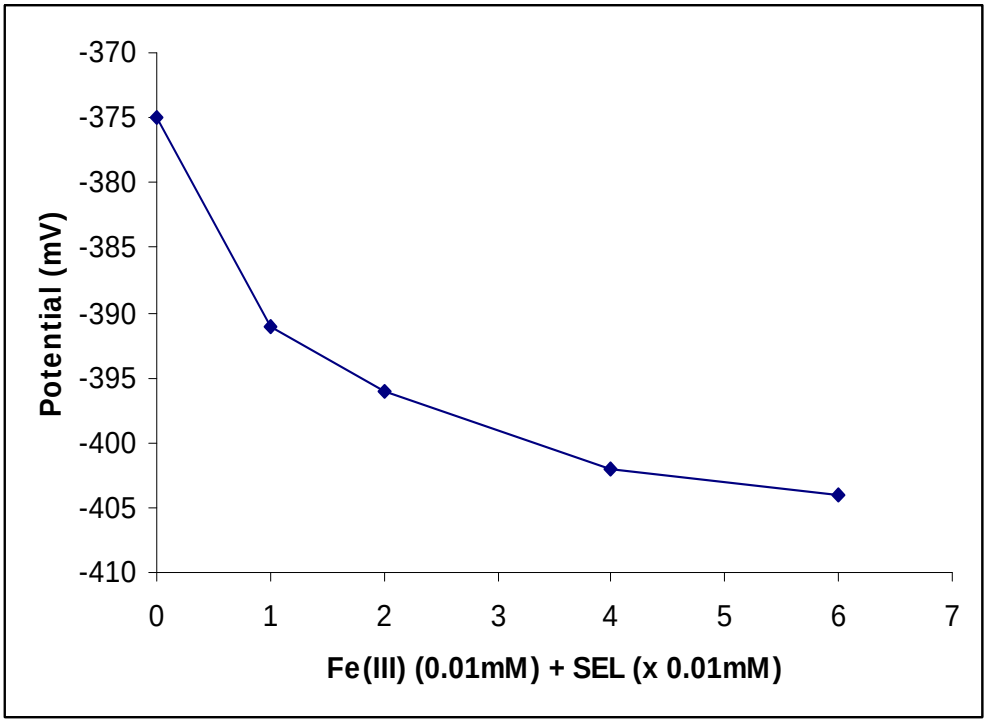


Figure 10.18: Effect of increasing concentrations of SEL on the reduction potential of the 0.01 mM Fe^{3+}.

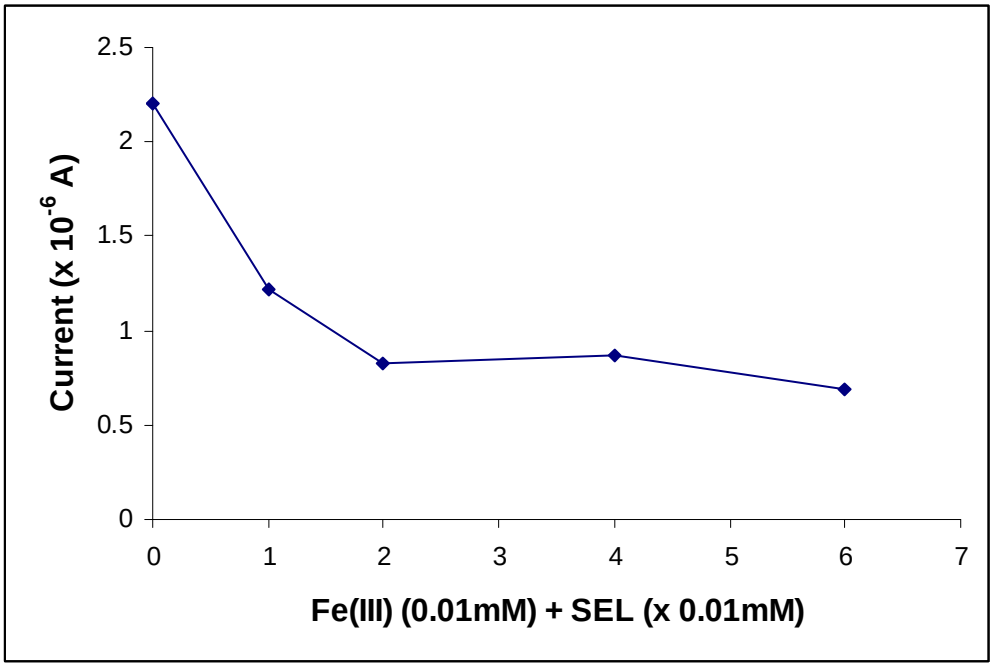


Figure 10.19: Effect of increasing concentrations of SEL on the peak current response of 0.01 mM Fe^{3+}.

10.5.4 DISCUSSION

The results of the present electrochemical study show that SEL has the ability to form a complex with Fe^{3+} which is more difficult to reduce , but easier to oxidize than Fe^{3+} alone and is therefore likely to inhibit the reduction of Fe^{3+} to Fe^{2+}. Figure 10.18 demonstrates that SEL has a concentration dependent effect on the decrease in reduction potential of Fe^{3+}. The binding of Fe^{3+} by SEL , may inhibit the reduction of Fe^{3+} to Fe^{2+} and provide another mechanism by which SEL can inhibit iron-induced lipid peroxidation (chapter 7), besides its direct scavenging properties (chapter 6).

Figure 10.19 illustrates that SEL causes a concentration dependent decrease in the current response of Fe^{3+}. This is expected since the Fe(III)-SEL complex has a more negative reduction potential and is consequently more difficult to reduce than the free metal ion, this in turn would hinder the cathodic current flow.

As mentioned previously, the acetylenic group (alkyne group) of the SEL molecule is the functional group most likely to be involved in iron binding. This group is important for the MAO-B inhibitory potency of SEL and further research should be done to investigate the effect of iron binding on the MAO-B inhibitory activity of SEL.

CHAPTER ELEVEN

11.1 SUMMARY OF RESULTS, CONCLUSIONS AND RECOMMENDATIONS FOR FUTURE STUDIES

11.1.1 SUMMARY OF RESULTS

CHAPTER 2: Stability of 6-Hydroxydopamine and its formation from dopamine

The stability of 6-OHDA under acidic, physiological and basic pH conditions was investigated in this chapter. 6-OHDA was most stable at an acidic pH (0.1 M $HClO_4$) and was very unstable under physiological (0.1 M PBS, pH 7.4) and basic pH (0.1 M NaOH) conditions. The stability of 6-OHDA was also dependent on ascorbic acid concentration. Ascorbic acid increased the stability of 6-OHDA. 6-OHDA was found to be most stable in 0.1 M $HClO_4$ containing 1 mM of ascorbic acid. The results of the stability study were used to minimize the loss of 6-OHDA during sample storage, homogenization and analysis in the experiments that followed. The results of this chapter also confirm the previously published observation that DA reacts with free •OH in *vitro* to produce the neurotoxin 6-OHDA. This conversion of DA to 6-OHDA *in vitro* is inhibited by SEL in a concentration dependent manner.

Production of 6-OHDA has been postulated to occur *in vivo*. The results of chapter 2 demonstrate that no detectable amount of 6-OHDA was found in the striatum after a single large dose of LD (50 mg/kg) or after acute LD administration (10 mg/kg/bd for seven days). The co-administration of LD (10 mg/kg/bd) with SEL (2.5 mg/kg/bd) for seven days also did not result in any detectable amount of 6-OHDA in the striatum *in*

vivo. In addition to this, an increase in striatal iron content prior to the administration of LD and SEL also did not result in the formation of detectable amounts of 6-OHDA in the striatum.

Chapter 3: Pineal indole metabolism

The aim of chapter 3 was to assess the effect of SEL on *in vitro* and *ex vivo* pineal indole metabolism. The results of the *in vitro* experiment demonstrate that SEL, at a concentration of 10 μM, increases the production of MEL and NAS by the rat pineal gland and results in a significant decrease in the monoamine oxidase products formed from 5-HT. The results of the *ex vivo* experiment demonstrate that the acute administration of SEL (2.5 mg/kg/bd) for seven days results in a significant increase in NAS levels, however, levels of MEL, 5-HTOH, 5-HIAA, 5-MTOH and 5-MIAA were not significantly altered. The resultant increase in pineal NAS levels following SEL administration could have implications in neuroprotection as NAS has been demonstrated to be a potent antioxidant in the brain. The rise in NAS induced by SEL in rats may be another mechanism by which this drug can protect against 6-OHDA formation and toxicity.

Chapter 4: Neurotransmitter levels

The aim of chapter 4 was to investigate the effect of LD and SEL on neurotransmitter levels in the striatum and hippocampus. LD treatment (10 mg/kg/bd for seven days) results in a significant increase in striatal and hippocampal DA, DOPAC and NE levels. Despite a two-fold increase in striatal DA levels, there was an absence of detectable levels of 6-OHDA in the striatum (chapter 2). Striatal and hippocampal levels of 5-HIAA and 5-HT were unchanged following LD administration. When used on its own, SEL (2.5 mg/kg/bd for seven days) results in an increase in striatal DA levels and a decrease in levels of DOPAC and HVA. This effect is attributed to the MAO-B inhibitory action of SEL. The effect of SEL on DA, DOPAC and HVA levels was limited to the striatum since the administration of SEL had no effect on DA, DOPAC and HVA levels in the

hippocampus. SEL treatment has no significant effect on striatal or hippocampal levels of NE, 5-HT or 5-HIAA. The elevation of NAS production by the pineal gland following SEL administration (chapter 3) can therefore not be attributed to inhibition of the MAO-mediated metabolism of NE or 5-HT. The increase in NAS after SEL administration is therefore most likely due to increased NAT activity. The co-administration of LD with SEL did not result in any significant change in striatal or hippocampal levels of DA or 5-HT compared to the levels of these neurotransmitters obtained with the use of LD alone. However, the co-administration of these drugs causes a decrease in striatal NE levels compared to the levels obtained with the use of LD alone.

Chapter 5: Superoxide anion formation

This chapter demonstrates that DA and 6-OHDA increase superoxide anion generation in whole rat brain homogenate *in vitro*. The increased generation of $O_2^{\bullet-}$ can be attributed to the formation of $O_2^{\bullet-}$ during the auto-oxidation of these compounds as well as the ability of DA and 6-OHDA to obstruct the flow of electrons along the mitochondrial electron transport chain and enhance the production of $O_2^{\bullet-}$ by increased electron leakage onto oxygen. The co-incubation of rat brain homogenate with DA and SEL demonstrates that SEL is able to abolish the rise in $O_2^{\bullet-}$ induced by DA. However, SEL has no effect on the increased production of $O_2^{\bullet-}$ induced by 6-OHDA. This may be due to the higher rate of auto-oxidation of 6-OHDA compared to DA. 6-OHDA should therefore be able to generate larger amounts of $O_2^{\bullet-}$ in a given time than DA. 6-OHDA is also a more powerful reducing agent than is DA and may obstruct electron flow along the mitochondrial electron transport chain by maintaining components of the chain in the reduced state. In addition to this, 6-OHDA is more easily converted to quinone oxidation products than is DA. DA-Q and 6-OHDA-Q can bind to thiol groups in mitochondrial proteins. Thiol cross-linkage of mitochondrial proteins is associated with mitochondrial dysfunction and increased ROS formation.

This chapter also demonstrates that LD treatment (10 mg/kg/bd for seven days) results in a significant increase in striatal $O_2^{\bullet-}$ production *in vivo*. The co-administration of SEL

(2.5 mg/kg/bd for seven days) with LD results in significantly lower striatal $O_2^{\bullet-}$ production compared to that observed in rats receiving only LD. The inhibition of $O_2^{\bullet-}$ production by SEL may be due to direct scavenging of $O_2^{\bullet-}$ by SEL or due to the ability of SEL to up-regulate $O_2^{\bullet-}$ metabolizing systems, particularly SOD.

Chapter 6: Hydroxyl radical formation

This chapter demonstrates that DA enhances •OH production by Fenton chemistry *in vitro*. Similar results were also previously obtained with 6-OHDA (Méndez-Álverez *et al*., 2001). In the presence of a mixture of Fe(II)-EDTA and H_2O_2, hydroxyl radicals are formed that react with salicylate to form 2,3-DHBA, 2,5-DHBA and CAT. These hydroxylated products of salicylic acid can be quantified using HPLC-ECD. The addition of DA to the Fenton system results in a concentration dependent increase in the formation of 2,3-DHBA, 2,5-DHBA and CAT. Therefore, despite the fact that DA can scavenge •OH to form 6-OHDA (neurotoxin), 5-OHDA and 2-OHDA, it also has the potential to enhance •OH generation under the same conditions. This effect is probably a consequence of the reported capacity of iron to catalyze the auto-oxidation of DA. In addition to this, the results of chapter 2 show that •OH can convert DA to 6-OHDA, a compound which has a higher rate of auto-oxidation than DA. The increased •OH production by DA is also dependent on the ascorbic acid concentration. Ascorbic acid causes an increase in the overall production of •OH by the Fenton system, which is most likely due to its ability to reduce Fe^{3+} to Fe^{2+}, however, when ascorbic acid is present at high enough concentrations it prevents the rise in •OH induced by DA. This is most probably due to the ability of ascorbic acid to prevent the auto-oxidation of DA and the resultant generation of •OH. SEL was able to inhibit the DA induced rise in •OH production by the Fenton system in a concentration dependent manner.

The aim of this chapter was also to investigate whether acute LD treatment (10 mg/kg/bd for seven days) results in an increased •OH production in the striatum. The results of the *in vivo* study found no evidence that LD treatment increases striatal •OH in the brain.

SEL treatment (2.5 mg/kg/bd for seven days) also has no effect on striatal •OH production *in vivo*.

Chapter 7: Lipid peroxidation studies

The results of this chapter show that the addition of Fenton reagents (Fe(II)-EDTA/H_2O_2 and ascorbate) to rat brain homogenate results in a marked increase in lipid peroxidation. DA and 6-OHDA cause a significant and concentration dependent decrease in the lipid peroxidation stimulated by the Fenton system *in vitro*. Some investigators postulate that the inhibition of lipid peroxidation by catecholamines is due to their ability to scavenge •OH and to chelate iron, thereby slowing or preventing •OH formation by the Fenton reaction. However, chapter 6 contradicts this hypothesis since it demonstrates that DA and 6-OHDA have the ability to enhance •OH production under similar conditions. The inhibition of lipid peroxidation by these compounds must therefore occur via a different mechanism. It is thought that the semiquinone radical formed during the auto-oxidation of these compounds may be preventing the propagation of lipid peroxidation by acting as a chain breaking antioxidant. SEL was also shown to inhibit lipid peroxidation induced by the Fenton system *in vitro*. However, much higher concentrations of SEL are needed to inhibit lipid peroxidation than DA or 6-OHDA. The combined action of DA and SEL enhances the reduction of lipid peroxidation by DA at low DA concentrations. However, when higher concentrations of DA were used, the combined action of DA and SEL actually resulted in higher levels of lipid peroxidation than the use of DA alone.

The *in vivo* study demonstrates that the intrastriatal injection of ferrous iron (10 nmol) results in a significant increase in striatal lipid peroxidation. The treatment of the Fe^{2+} infused rats with LD, SEL or a combination of these drugs results in a significant decrease in the Fe(II) induced lipid peroxidation. However, there was no significant difference in lipid peroxidation levels between the LD treatment group and the group that received LD and SEL.

Chapter 8: Protein oxidation studies

The results of this chapter demonstrate that the incubation of rat brain proteins with Fenton reagents causes a significant increase in protein carbonyl content. The addition of DA or 6-OHDA to incubations also causes a significant increase in the carbonyl content of rat brain proteins. The increase in carbonyl content is greater for 6-OHDA than DA. The combined action of (DA + Fenton system) and (6-OHDA + Fenton system) significantly augments the carbonyl content obtained with the Fenton system alone. This increase in carbonyl content is dependent on the concentration of DA or 6-OHDA used. The protein oxidation induced by DA and 6-OHDA could be related to free radical production during the auto-oxidation of these molecules or covalent binding of DA-Q and 6-OHDA-Q to the protein. SEL is unable to inhibit the protein oxidation induced by 6-OHDA and DA. This finding suggests that the quinone oxidation products of DA and 6-OHDA rather than ROS are involved in the protein oxidation induced by these compounds. SEL has been shown to inhibit the production of ROS by DA and 6-OHDA (chapter 5 and 6).

The *in vivo* study demonstrates that the intrastriatal injection of ferrous sulfate (10 nmol) results in a significant increase in striatal protein oxidation. However, the results of the study do not support the hypothesis that an increase in striatal DA concentration produces oxidative damage to proteins because the i.p. administration of LD (10 mg/kg/bd for seven days), SEL (2.5 mg/kg/bd for seven days) or a combination of these drugs has no significant effect on iron-induced protein oxidation.

Chapter 9: Total glutathione content

Chapter 9 demonstrates how the incubation of rat brain homogenate with Fenton reagents causes a significant reduction in the total GSH content of the homogenate. Incubation of rat brain homogenate with DA or 6-OHDA also causes a significant decrease in total GSH content. The combined action of (DA + Fenton system) and (6-OHDA + Fenton system) causes a greater reduction in total GSH content when compared with the values

obtained for the Fenton system alone. The decrease in the thiol content is most likely due to the ability of DA-Q and 6-OHDA-Q to bind to the thiol groups of the GSH molecules. SEL is unable to inhibit the reduction of total GSH induced by the combined action of (DA + Fenton system) or (6-OHDA + Fenton system).

The *in vivo* study shows that the intrastriatal injection of Fe^{2+} causes a significant decrease in the total GSH content of the striatum. This decrease in GSH is not attenuated or enhanced by treatment with LD (10 mg/kg/bd for seven days), SEL (2.5 mg/kg/bd for seven days) or a combination of these drugs.

Chapter 10: Iron interaction studies

The results of the ferrozine assay demonstrate that DA and 6-OHDA, even when used at relatively high concentrations are unable to chelate Fe^{2+} and inhibit the formation of the purple Fe(II)-ferrozine complex. This chapter also demonstrates that SEL has weak Fe^{2+} chelating activity and produces a 20 % inhibition of Fe(II)-ferrozine complex formation when used at concentrations ranging from 0.025 – 1 mM. The results of this chapter also confirm the previously published observation that DA and 6-OHDA are capable of releasing iron, as Fe^{2+} from ferritin. This iron is therefore in the low valence state necessary for participation in the Fenton reaction. 6-OHDA is more effective at releasing iron from ferritin than DA. This is thought to be a consequence of the redox potential of 6-OHDA. The AdSVs from electrochemical analysis demonstrate that both DA and SEL have the ability to bind Fe^{3+} to form a metal-ligand complex that is more difficult to reduce than Fe^{3+} itself. DA and SEL are therefore likely to curtail the conversion of Fe^{3+} to Fe^{2+}, the form of iron required for the Fenton reaction. The chelation of Fe^{3+} by DA suggests that the increase in •OH production on addition of DA to the Fenton system (chapter 6) is probably not due to the involvement of Fe^{2+} in the production of •OH by the Fenton reaction. Thus, the observed effect is probably a consequence of the reported capacity of Fe^{3+} to catalyze the auto-oxidation of DA, leading to increased •OH production. The results of the AdSV of 6-OHDA were inconclusive since the 6-OHDA peak interfered strongly with the peak for Fe^{3+}. For this reason cyclic voltammetry was

used to investigate whether there is any interaction between Fe^{3+} and 6-OHDA. The CV results provide no evidence for the formation of a 6-OHDA-Fe(III) complex and support the hypothesis that 6-OHDA interacts with Fe^{3+} via an outer sphere electron transfer reaction (Linert *et al*., 1996).

11.1.2 CONCLUSIONS

Several hypotheses exist which attempt to explain the loss of nigrostriatal dopaminergic neurons in PD. One theory proposes that nigral neurons are selectively vulnerable to environmental and/or endogenous neurotoxins that cause mitochondrial dysfunction. This in turn leads to impaired energy metabolism and neuronal death (Orth and Schapira, 2002). Secondly, the proteolytic stress hypothesis ascribes the loss of nigral neurons in PD to the toxic accumulation of misfolded and aggregated proteins (McNaught and Olanow, 2003). Lastly, the preferential loss of nigrostriatal neurons in PD has been attributed to the highly oxidative intracellular environment in dopaminergic neurons (Lotharius and Brundin, 2002).

This research project provides some evidence that DA acts as an important vulnerability factor in PD. The results of the *in vitro* studies suggest that DA is a double-edged sword which, on the one hand protects against the propagation of lipid peroxidation (chapter 7), but on the other hand enhances the generation of toxic oxyradicals, such as the superoxide radical anion (chapter 5) and the extremely cytotoxic hydroxyl radical (chapter 6). DA can also be converted to the potent neurotoxin, 6-OHDA (chapter 2), promote the oxidation of rat brain proteins (chapter 8) and reduce the total GSH content of rat brain homogenate (chapter 9). Furthermore, these properties are enhanced in different manners by the presence of Fenton reagents. Chapter 10 also demonstrates that DA is capable of releasing iron, as Fe^{2+} from ferritin. This most likely occurs via a superoxide dependent mechanism and increases the fraction of iron available for interacting with H_2O_2 and producing •OH.

The increase in superoxide production by DA may be due to the ability of DA to cause mitochondrial dysfunction by inhibiting important mitochondrial complexes in the mitochondrial electron transport chain (Glinka and Youdim, 1995). DA has also been shown to form covalent, oxidative adducts with proteins. This may be the underlying mechanism involved in the protein oxidation induced by DA. It has also been suggested that DA can cross-link proteins by reaction with two cysteinyl residues (Graham *et al*., 1978). This would promote protein aggregation and the formation of Lewy bodies in the cell. One of the consequences of the decrease in GSH induced by DA is that it may indirectly stimulate lipid peroxidation by weakening the endogenous GSH-dependent detoxification of H_2O_2 and other ROS (Boots *et al*., 2002). These findings contribute to explain the progression of PD due to the reported increase in DA turnover in surviving dopaminergic neurons. The *in vitro* experiments with 6-OHDA demonstrate that this neurotoxin behaves very similarly to DA. 6-OHDA is however more effective at causing oxidative stress (an increase in ROS production), iron release from ferritin, protein oxidation and a decrease in total GSH levels than is DA.

The pro-oxidant properties of DA may be well-controlled by the endogenous antioxidant defense systems against oxidative stress in dopaminergic neurons. However, problems arise with high concentrations of DA, because of the possibility that the resulting oxidative stress cannot be controlled by the corresponding defense systems, a fact that may be exacerbated by exogenous LD administration. If this hypothesis is correct, administration of exogenous LD might lead to accelerated dopaminergic neuronal decline in PD patients. This in turn would hasten the emergence of wearing off effects associated with LD therapy. Wearing off effects are attributed to reduced interdose presynaptic DA storage capacity (Chase *et al*., 1993; Nutt and Holford, 1996). The results of the *in vivo* studies conducted in this research project demonstrate that the exogenous administration of LD (10 mg/kg/bd for seven days) to normal (unlesioned) male Wistar rats results in an increase in striatal superoxide anion production, however studies found no evidence that LD administration increased striatal concentrations of hydroxyl radicals. It has been postulated that although LD is not toxic to normal dopaminergic neurons in rats, LD may be toxic to neurons in partially damaged striatum. In an attempt to address this issue rats

received an intrastriatal injection of ferrous iron before they were treated with LD for one week (seven days). Striatal samples were then analysed for lipid peroxidation, protein oxidation and GSH content. Despite the increase in striatal iron levels, LD treatment was not toxic and had no detrimental effect on lipids, proteins or GSH. LD administration inhibited iron-induced lipid peroxidation. The *in vitro* toxicity of DA therefore does not correlate well with the apparent lack of toxicity of LD *in vivo*.

This research project also demonstrates that SEL, a selective MAO-B inhibitor is able to protect against some of the pro-oxidant effects of DA and 6-OHDA *in vitro* and LD treatment *in vivo*. In addition to its MAO-B inhibitory activity this study has also shown that SEL possesses antioxidant and metal binding properties. SEL inhibits the formation of superoxide radicals induced by DA *in vitro* and by exogenous LD administration *in vivo*. The results of the research also demonstrate that SEL has the ability to inhibit hydroxyl radical formation via Fenton chemisty and DA auto-oxidation in a cell free environment. The decrease in hydroxyl radical generation can therefore not be attributed to its MAO-B inhibitory activity. SEL inhibited iron-induced lipid peroxidation *in vitro* and *in vivo* and combined treatment of rats with LD and SEL resulted in a more significant decrease in iron-induced lipid peroxidation than those rats treated with LD alone. SEL did not however have any effect on protein oxidation or the loss of GSH induced by DA, 6-OHDA or the Fenton system *in vitro*.

Another conclusion drawn from the research is that the oxidation of biomolecules, particularly lipids and proteins must proceed via different mechanisms. If the oxidation of lipids and proteins occurred via the same mechanism then their oxidation would be stimulated or inhibited by the same chemicals. This is not the case since DA and 6-OHDA inhibit lipid peroxidation, but stimulate protein oxidation.

Treatments aimed at preventing the death of dopaminergic neurons in PD depend on a better understanding of the mechanisms that control neuronal cell death in PD.

11.1.3 RECOMMENDATIONS FOR FUTURE STUDIES

The short duration of LD therapy (one week) to rats in the present study does not provide sufficient evidence to draw reliable conclusions concerning the loss of efficacy and the long term side effects derived from the treatment of Parkinson's disease with LD. Future research needs to be conducted on the effects of chronic LD treatment (for at least 6 months) on the ability of LD to produce oxidative stress (an increase in the production of ROS) and oxidative damage to important biological molecules such as lipids and proteins.

Although the results of the current research do not support the hypothesis that LD is toxic to dopaminergic neurons, additional factors need to be considered in the treatment of PD with LD. The current study focuses mainly on increased striatal iron concentrations. However, other factors associated with PD such as reduced mitochondrial enzyme complex I activity and GSH deficiency may also increase the likelihood of oxidative stress and oxidative damage after LD therapy. These factors require further investigation.

Chapter 2 demonstrated that no detectable amount of 6-OHDA was found in the striatum of rats despite an increase in striatal DA and iron levels. It was postulated that rapid scavenging of oxidized 6-OHDA by the sulfhydryl groups in GSH, albumin and other proteins may account for the absence of striatal 6-OHDA. To confirm this explanation further research needs to be conducted to demonstrate the presence of 6-OHDA conjugated to protein in the striatum of rats receiving LD treatment.

Chapter 3 demonstrates that the administration of SEL to rats results in a significant increase in the production of NAS by the pineal gland. The results of chapter 4 show that this effect cannot be attributed to an inhibition of 5-HT metabolism or due to an inhibition of NE degradation. It was postulated that SEL may enhance the activity of N-acetyltransferase (NAT), the enzyme responsible for the conversion of serotonin to NAS. Further research needs to be conducted in order to investigate the effect of SEL on the activity of NAT.

The results of chapter 7 demonstrate that both DA and 6-OHDA inhibit lipid peroxidation induced by the Fenton system *in vitro*. Some researchers have attributed this to the ability of these compounds to either scavenge free radicals or bind iron. However, the ability of DA and 6-OHDA to enhance •OH production under similar experimental conditions contradicts this hypothesis. Another area for future research would therefore be an investigation into the mechanism by which these agents inhibit lipid peroxidation. LD administration also reduces iron-induced lipid peroxidation *in vivo*. The mechanism through which this inhibition is achieved should be investigated further.

Further research should be conducted to confirm whether the quinone oxidation products of DA and 6-OHDA or the oxygen free radicals formed during their auto-oxidation are involved in the protein oxidation and loss of total GSH induced by these compounds. The effect of DA and 6-OHDA on specific proteins, e.g. α-synuclein requires further investigation.

Chapter 10 shows that SEL possesses iron binding properties. The effect of iron binding by SEL on the MAO-B inhibitory activity of this compound is another avenue for future research.

APPENDIX ONE

Housing of Animals

All the work involving the use of animals was approved by the Rhodes University animal ethics committee. Adult male rats of the Wistar strain, weighing between 250-300g were used throughout the study. The animals were purchased from the South African Institute for Medical Research (Johannesburg, South Africa). Rats were housed in a controlled environment with a 12 hour light:dark cycle, and were given access to standard laboratory food and water *ad libitum*. The animal room was windowless with automatic temperature and lighting controls. The temperature of the animal room was maintained between 20 ºC and 25 ºC while an extractor fan ensured the constant removal of stale air. The cages were cleaned daily.

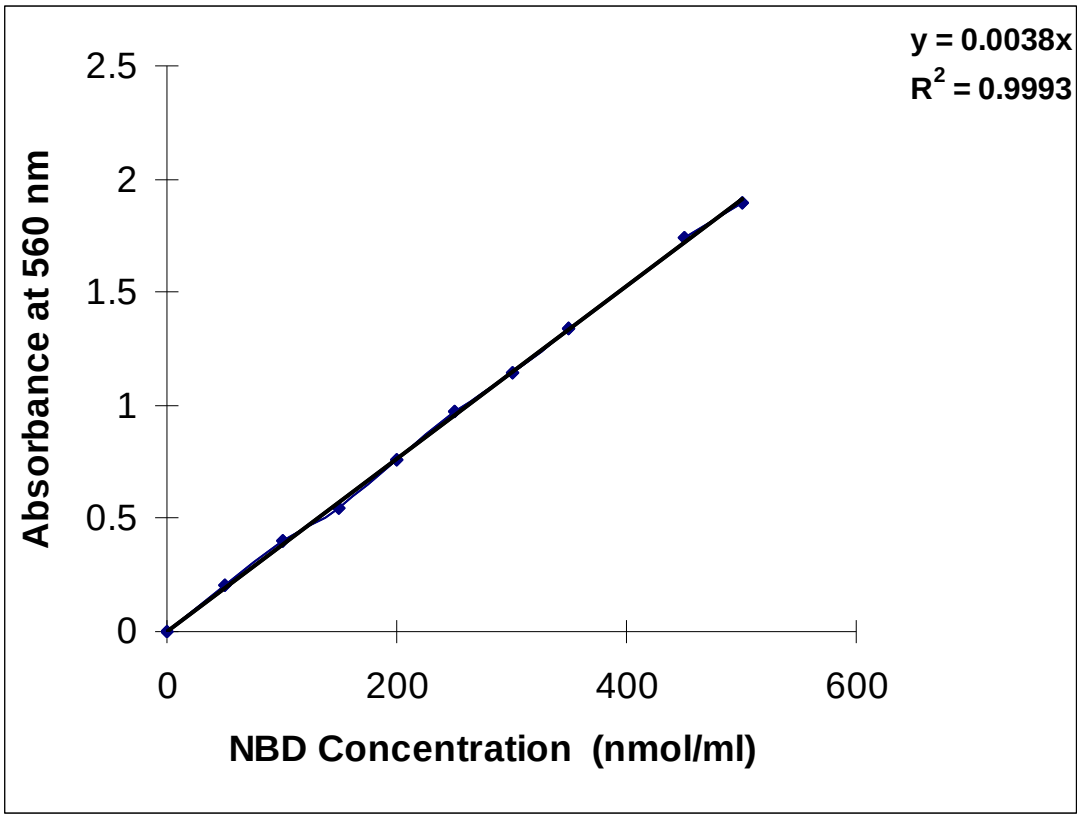
APPENDIX TWO

Sacrificing and dissection of the animals

Rats were sacrificed swifly by cervical dislocation and rapidly decapitated. To remove the brain, the top of the skull was removed and the brain was exposed by making an incision through the bone on either side of the pariental suture, from the foramen magnum to near the orbit. The calvarium was removed, exposing the brain, which was easily removed for use in experiments. The brains were either used immediately or stored at -70 ºC until used.

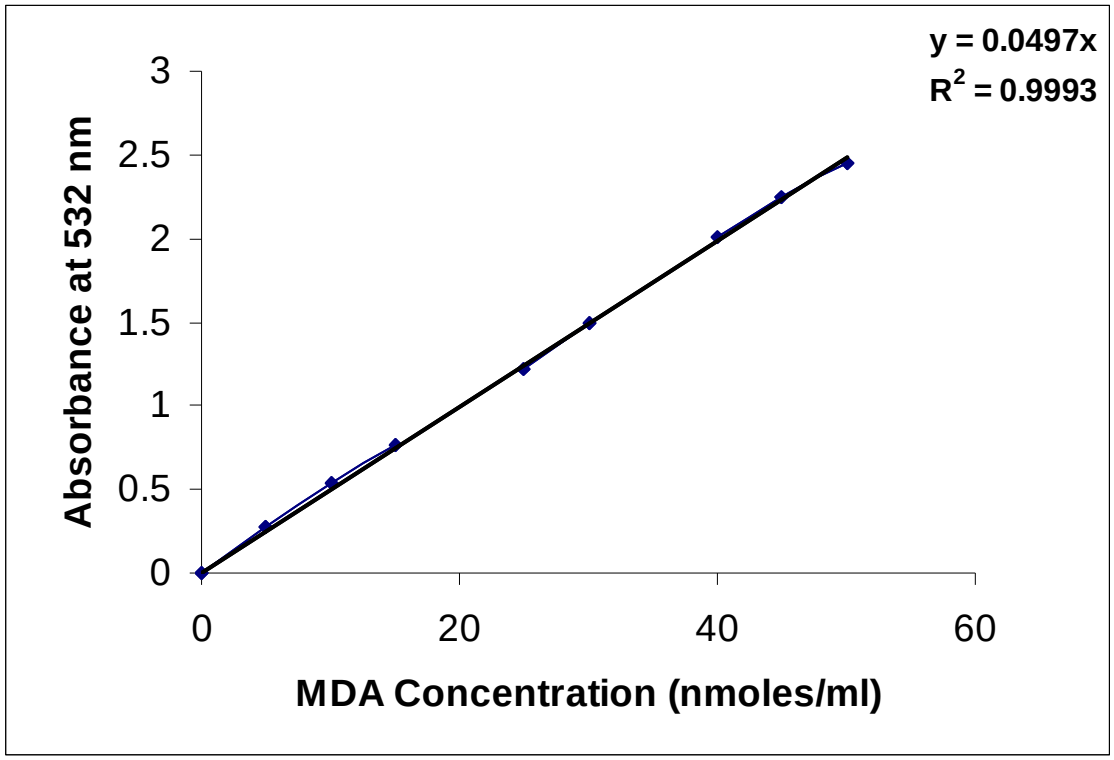
When necessary, the striatum was dissected out from each hemisphere of the whole brain, rapidly frozen in liquid nitrogen and stored at -70 ºC until used.

APPENDIX THREE



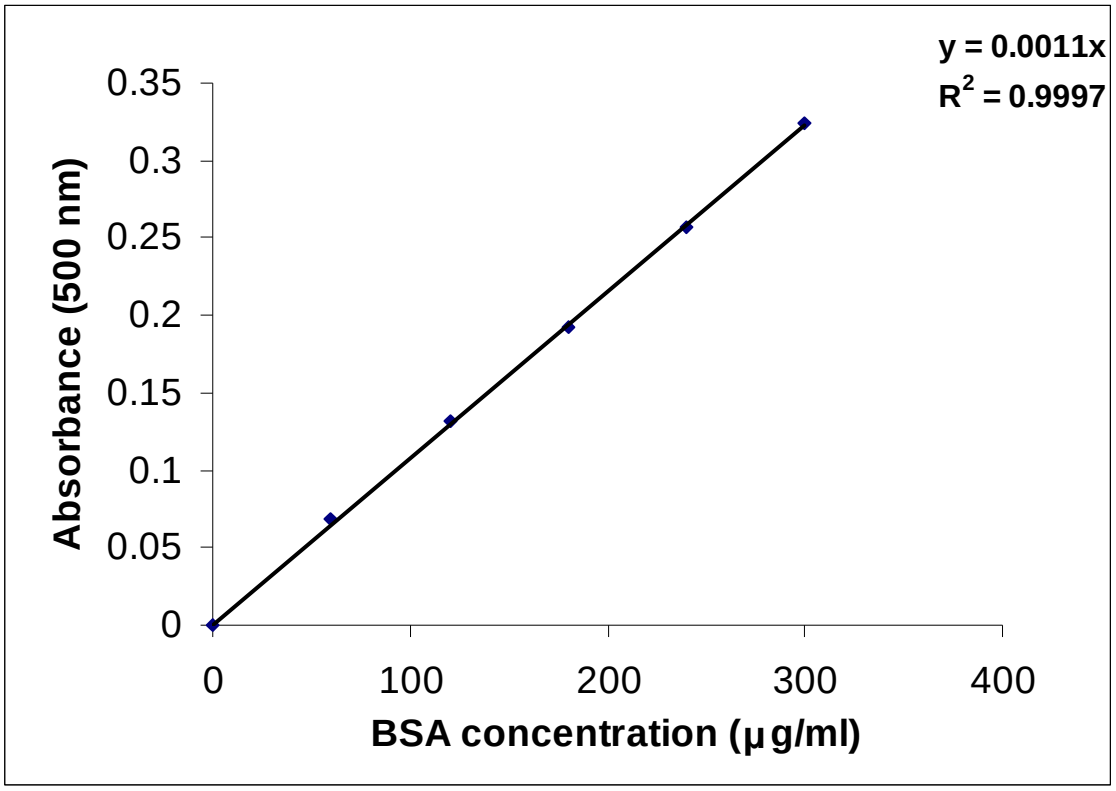
NBD Standard Curve

APPENDIX FOUR



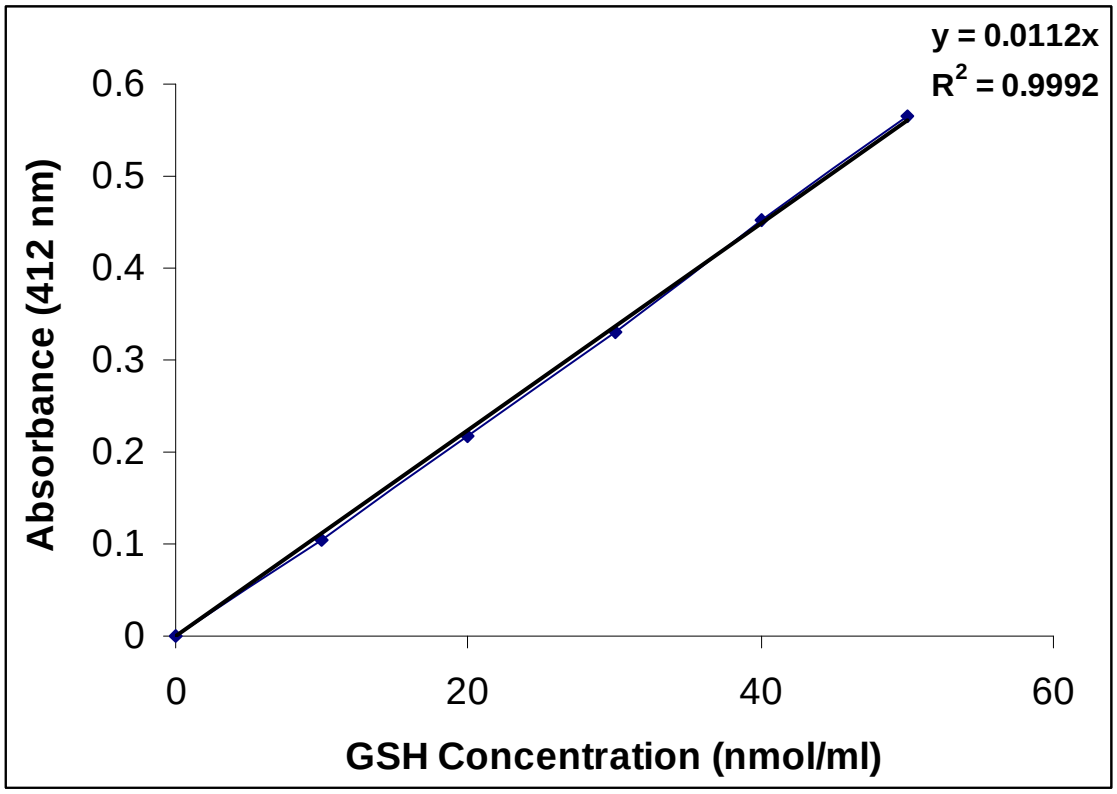
MDA Standard Curve

APPENDIX FIVE



Protein Standard Curve

APPENDIX SIX



GSH Standard Curve

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